THE UNIVERSITY OF ALBERTA

THE EFFECT OF PRETREATMENT WITH ANTIDONOR ANTISERA AND ANTIBODY FRAGMENTS ON RENAL AND SKIN XENOGRAFTS REJECTION

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The effect of pretreatment with antidonor antisera and antibody fragments on renal and skin xenografts rejections submitted by Mohammad Bakri bin Musa in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The rejection of xenografts, like that of allografts, is immunologically mediated. Grafts exchanged between closely related species are rejected primarily via cell-mediated immunity; those between widely divergent species by humoral antibodies (hyperacute rejections with vascularised organs and "white grafts" reaction with skin). Humoral-mediated rejections are seen with allografts in presensitised hosts.

The sequence in the humoral-mediated rejection is thought to be as follows. The preformed antibodies combine with graft antigens, triggering the chain of reactions involving complement leading to cellular destruction. With vascular grafts there is associated local consumption of formed blood elements and various coagulation factors. The release of vasoactive compounds may play a subsidiary role. The pepsin-digested antibody fragment $F(ab)_2$ retains its antigen-binding capacity, but devoid of the Fc fragment is unable to fix complement. Antigen-antibody reaction involving the $F(ab)_2$ does not lead to subsequent cellular damage; such combinations however can mask or protect the antigenic sites from subsequent attack by the intact antibody or immune lymphocytes. Thus pretreating grafts with their specific $F(ab)_2$ has been shown to be effective in delaying the hyperacute and cell-mediated rejections of dog and monkey kidneys and with tumor xenografts of Guinea pigs.

In this experiment an attempt is made to extend the observation to renal and skin xenografts. Rabbit kidneys were perfused for thirty minutes with the pepsin-digested antidonor antisera (ADS) (dog-anti-rabbit) prior to transplanting them to the dog. The ADS were prepared by immunising the dog with the prospective donor lymphocytes in complete Freund's adjuvant. The survival of these grafts were compared to those pretreated with the

intact antisera, control antisera and their $F(ab)_2$ and saline. Similarly, C57BL/6 mouse skin was incubated with the ADS $F(ab)_2$ (rat-anti-mouse) prior to transplanting them onto Lewis rats, and the survival compared to those in the control groups. The ADS were prepared by immunising Lewis rats with C57BL/6 mouse spleen cells and homogenised skin with the adjuvant.

With the renal xenografts, pretreatment with the pepsin-digested ADS did not delay their rejections. The kidneys in all the experimental groups were rejected within ten minutes. With the skin xenografts, pretreatment with both the pepsin-digested and intact ADS were effective in delaying their rejections. The mean survival times (MST) for these two groups were 7.22 ($\pm$0.97) and 7.36($\pm$1.43) days respectively compared with 5.93 ($\pm$0.83) days with the saline-treated group ($p < 0.005$). Further, the skin grafts treated with the ADS and their $F(ab)_2$ were rejected with cellular infiltration, after being vascularised, akin to a CMI reaction. In contrast, the control grafts were rejected as "white grafts" - not vascularised and with minimal cellular infiltration, that is humoral-mediated.

The failure to prolong the renal xenografts may be attributed to the inappropriate technique of preparing the ADS, the overwhelming hyperacute rejection or the instability of the antigenic masking by the $F(ab)_2$. The efficiency of both the intact ADS and their $F(ab)_2$ in prolonging the skin xenografts suggests that the ADS prepared by the particular technique act *in vivo* as a non-cytotoxic and blocking antibody. Further by blocking the action of the humoral-mediated rejection the role of CMI can be demonstrated in the rejection of skin xenografts.

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I NOTES ON TERMINOLOGY

The terminology used in this thesis conforms to that suggested by Gorer (1961). The terms commonly used are briefly defined here:

a) Autoplastic grafts (autografts) are those confined within the same individual. The factors determining the survival of such grafts are non-immunological.

b) Syngeneic grafts are grafts that are transplanted into a genetically identical recipient. Such grafts will be expected to behave like autografts. Clinically, this is seen in grafts between identical twins and, experimentally, in grafts between members of highly in-bred animals.

c) Grafts between individuals within the same species, excepting syngeneic pairs, are termed allografts. Immunological factors largely determine the survival of such grafts.

d) Grafts exchanged between members of different species are called xenografts. Where the species are closely related the pattern of rejection resembles those of allografts. Grafts between widely disparate species however are rejected more vigorously, reflecting the wider genetic disparity.

An organ transplanted into a location similar to that from where it was taken is said to be 'orthotopically' transplanted (e.g. skin placed on the surface of the body); it is 'heterotopically' transplanted if placed in an unnatural location (as with skin

implanted subcutaneously).

II HOST RESPONSE TO ALLOGENEIC GRAFTS

Before attempting to study cross-species transplantation, a review of the normal allograft response seems appropriate. In this section the sequence of events comprising the allograft rejection process and the basis for this reaction will be discussed.

A) Genetics of Transplantation and Transplantation Antigens.

Transplants of living tissues between adult animals do not usually survive and function indefinitely. It was noted very early that this incompatibility of grafting is a function of the genetic disparity between the donor and recipient. Clinically it was noted that the success of skin grafting could be directly correlated with the degree of genetic similarity between the donor and recipient. Experimentally, grafts between members of one syngeneic strain were accepted indefinitely while those exchanged between different strains were rejected (Ceppellini 1968). Further the F_1 hybrid from two genetically different parental strains would accept grafts from either parental strains; the reverse, that is F_1 to parental grafts were rejected. Thus reactivity to a graft depended not on genetic similarity between host and graft but on whether the grafts have antigens that are absent in the host and capable of evoking the host's immune response (Snell 1966). These antigens, which are the end products of multiple genes are called 'histocompatibility' or 'transplantation' antigens (Terasaki 1971). The transplantation antigens of the mouse and man are the two that have been widely studied thus far.

In the mouse the specificities of these antigens are under the control of genes at a large number of loci. Of these the most important in tissue transplantation is the H-2 locus, which has over two dozen allelic forms (Davies 1968). The H-2 antigens are present in all cells and are especially well represented in lymphoid tissues. They appear very early in ontogeny and are associated with cell-surface membrane and intracellularly with the endoplasmic reticulum (Kahan 1969). As these genes are co-dominant, both the allelic forms in an individual are expressed with each parent contributing one allele. All the histocompatibility loci are believed to be located on the autosomes. In some strains of mice and rats however, some minor histocompatibility loci are associated with the sex chromosomes so that animals of the same strain but different sexes are not genetically identical with respect to transplantation antigens (Snell 1966).

In humans, strong barriers to transplantation are determined by the Human Leucocyte-A and blood group (AB) antigens (Russell 1970). The HL-A antigens are analogous to the H-2 of the mouse excepting that they are absent from the erythrocytes. The HL-A locus is now regarded as being made up of two closely linked sub-loci - the first governs the antigens HL-A 1, 2, 3, 9, 10, and 11 while the second the antigens HL-A 5, 7, 8, 12 and 13. Each child thus receives one chromosome containing the two genes, governing the specificities of the first and second series, from each parent (Terasaki 1971). These transplantation antigens become manifested only during grafting, however their presence can be detected indirectly by various serologic tests (platelet and

leucoagglutination, lymphocytotoxicity) and the mixed lymphocyte culture. Since these antigens are capable of evoking strong allograft reaction, considerable attempts are made at isolating and identifying their physico-chemical structure. Current thinking is that these antigens are water-insoluble lipoprotein complex containing equal portions of lipids and proteins and a low carbohydrate content (Kahan 1969). The AB antigens are of course the polysaccharide secreted on the red cell wall (Humphrey 1970).

B) The Nature of the Allograft Rejection Process

An allogeneically transplanted skin will initially heal like an autograft and becomes invaded by host's blood vessels and lymphatics. After about a week the graft will be infiltrated by mononuclear cells and the blood flow in the new vessels becomes sluggish, leading to thrombosis and subsequent necrosis and sloughing of the graft. This sequence of events, which takes about two weeks to complete, is referred to as the 'first set' allograft reaction. If, after a latent period of one to two weeks after the rejection of a first set graft, a second graft from the same donor is applied, the same sequence of events is again noted. This time however the tempo of the rejection is much accelerated, taking less than a week - the 'second set' phenomenon (Medawar 1944). A graft from another donor however will be rejected in the same manner as the first set, that is, the second set reaction is a specific one. If the latent period between the rejection of the first set graft and the application of the second graft is shortened to within a week, a special form of rejection, the 'white graft' reaction is noted. It is so called

because the graft is characterised by pallor and lack of revascularisation (Rapaport 1958). The white graft reaction is a reflection of the heightened sensitivity in the few days following the rejection of a graft.

First set renal transplant, provided it is not badly damaged technically, will begin to secrete urine soon after revascularisation. After about a week it gradually ceases to function and becomes swollen and edematous, and on histologic sections shows diffuse infiltration by mononuclear cells (Dempster 1953). Second set renal transplant is rejected more dramatically and often fails to function completely (Calne 1963, Egdahl 1955). Calne described that following the release of vascular clamps the kidney became purplish and flaccid, and there was very little venous outflow. Histologically, it showed multiple cortical hemorrhages and edema but surprisingly little cellular infiltrate. This dramatic destruction of the kidney as a consequence of prior sensitisation is referred to as 'hyperacute rejection'.

There is now little doubt that the rejection of an allograft is an immunologically-mediated process. The evidences to support this conclusion are outlined below:

a) A latent period exists before an allograft is rejected. This latency implies that the rejection is an adaptive response of the host to the graft. Further, second set grafts are rejected faster and that this second set phenomenon is a systemic response and is specific for the donor (Medawar 1944).

b) Various antibodies directed against the donor tissues are detected following transplantation (Stetson 1963).

c) By interfering with the immune response of the host, the graft survival can be prolonged (Russel 1965).

d) Allograft immunity may be readily transferrable via transfer of lymphocytes or antibody.

There are two aspects in the immune response of the host to an allograft - first is the recognition of 'foreigness' and second, the marshalling of the various immune 'effector mechanisms' of the host leading to destruction of the graft.

(i) Sensitisation of Host by Graft

The primary cell responsible for initiating the allograft reaction is the lymphocytes - in particular, the small, long-living, thymus-dependent lymphocytes that freely circulate between the vascular and lymphatic channels (Gowan 1965). Following contact with the appropriate antigens, in vitro or in vivo, these cells enlarged, became pyroninophilic and undergo mitoses. During this 'blastic transformation' they released a number of factors ('lymphokines') one of which, the blastogenic factor, would stimulate other cells to undergo similar transformation. Some of these activated cells would take part as 'killer cells' to effect graft destruction, others differentiate to become 'memory cells' and a few may co-operate with the marrow (B) lymphocytes that will differentiate into antibody-forming cells (Roitt 1969). A few days following skin grafting these blast cells are seen in the regional nodes and at the graft site (Scathorne 1957). The host's lymphocytes may become sensitised through direct contact with the graft antigens either at the host-graft interphase or when circulating in the new blood vessels of the graft. These sensitised cells then migrate, via the lymphatics, to

the regional nodes where they undergo blastogenesis and recruit other cells to undergo similar changes (Wilson 1967). The findings that grafts in areas not drained by lymphatics (meninges, hamster's cheek pouch and skin flaps with only vascular attachments) survived for prolong periods seem to emphasise the importance of lymphatic continuity for sensitisation.

With kidney grafts we have immediate vascular continuity between host and graft so that the lymphocytes may become sensitised in circulating through the kidney (Strober 1965). In addition there may be actual shedding of the transplantation antigens into the vascular stream and these are picked by the circulating lymphocytes (Najarian 1966). Sensitisation via the lymphatic route however is minimal (Hume 1955).

(ii) Role of Cellular Immunity

The two classical immunological responses - cell-mediated immunity (CMI) and the production of humoral antibody - are both seen following allogeneic grafting. However the current view is that most instances of allograft rejection (the white graft and hyperacute rejection are two exceptions) are an expression of cell-mediated immunity. Thus allograft immunity may be readily transferable via lymphoid cells and can be manifested as the typical tuberculin-type skin reaction (Russel 1965). A more direct demonstration of the destructive ability of lymphocytes was provided by the work of Elkin (1964). Using rats, he showed that sensitised and non-sensitised lymphocytes from the parental strains when injected into the kidneys of their F_1 progeny can effect renal damage, while the sera (immune and non-immune) and lymphocytes from tolerant parents could not.

Similarly, lymphocytes of dogs that had rejected a kidney could destroy in vitro target kidney cells from the same donor (Govaerts 1960).

Although the primary role of lymphocytes in the allograft response is well established, the question remains as to how exactly these cells effect graft destruction and what governs their specificity. The suggested possibilities are that these cells synthesise antibody (hence the specificity) when in contact with the target cells or they may carry on their surfaces, antibody synthesised elsewhere (Wilson 1967). The possibility that the ultimate effectors may not be an antibody cannot be ruled out. It is known that activated lymphocytes released a number of factors (blastogenic, macrophage inhibiting and transfer factors) and these may participate in graft destruction (Wilson 1967).

(iii) Role of Humoral Antibody

The role of humoral antibody in allograft immunity has recently been reviewed by Stetson (Stetson 1963). Antibody can greatly influence the fate of the graft - either prolonging its survival or markedly accelerating its destruction, depending upon the graft system and the specie involved. With lymphoid grafts, the presence of anti-donor antibody usually means destruction of the grafts; further, immunity to bone marrow grafts may be transferable via the serum (Gorer 1959). With tumour cell grafts, they may either be susceptible or resistant to the effect of antibody. The susceptibility of these tumour grafts depends, among other things, on the concentration of the antigens on their surfaces and the accessibility of these surface antigens to antibody (Russel 1970). Paradoxically

in some instances, graft survival was actually prolonged in the presence of antibody. This special phenomenon (enhancement) will be discussed later. With skin grafts, humoral antibodies are involved in the Arthus-like reaction that characterises the 'white graft' reaction (Stetson 1963). Antibody may also play a role in the accelerated second set reaction but attempts at transferring this immunity via serum had been successful in only a few instances and under special experimental conditions (Steinmuller 1962, Stetson 1958). Essentially the special condition required being an extremely high concentration of the antibody at the graft site. Interestingly, skin grafts on agammaglobulinemic patients survived for months as compared to only a couple of weeks in normal recipients (Good 1957).

The presence of pre-existing antidonor antibody in kidney transplant recipients is usually associated with a rapid and dramatic destruction of the graft (hyperacute rejection). Clinically, this is seen in recipients receiving kidneys from ABO incompatible donors (the antibody being the anti-A and/or anti-B isoagglutinins) (Starzl 1964) and also from ABO compatible donors (Terasaki 1965, Kissmeyer-Neilsen 1966). The antibody in this latter instance was probably produced in response to an earlier sensitisation - through multiple blood transfusions, multiparity or previous transplants. This phenomenon of hyperacute rejection of kidney allografts had been investigated extensively in laboratory animals. The usual technique for sensitisation is to exchange repeated skin grafts between the donor and recipient, followed by the kidney transplant. Variable titers of various antidonor antibodies (lymphocytotoxins, heme- and leuco-agglutinins) were detected following this sensitisation. Renal

transplants in such a presensitised host invariably failed to function, rapidly become cyanotic, mottled and congested followed by a rapid decline in their blood flow. Rejection is completed within hours (Boehmig 1971). In addition, such grafts act as a trapping mechanism for the sequestration of formed blood elements and the adsorption of various antibodies. Local and sometimes systemic changes in various coagulation factors may be detected (Starzl 1970, Boehmig 1971). Like other humoral-mediated immune responses, the release of vasoactive compounds (histamine and serotonin) has also been observed (Rosenberg 1969). Further the rapidity of the rejection could not be correlated with any of the antibody titers. This lack of predictive value of the commonly measured antibodies seems to agree with the clinical observation that hyperacute rejection is occasionally seen in patients with no detectable antidonor antibody as currently measured (Starzl 1968).

One postulated mechanism for this hyperacute rejection is that the preformed antibody becomes attached to the endothelium of the renal vasculature initiating the binding of complement with subsequent endothelial damage, and the aggregation of white cells (especially the polymorphs) and the formation of microthrombi of platelets and other formed elements - in effect a local Arthus reaction in the graft (Williams 1968). Starzl (1970) however was more impressed by the histologic features of such grafts, in particular, the deposition of fibrin-like material in the glomeruli and arterioles together with the systemic changes in coagulation factors seen in some of the experimental animals and patients, and suggested that these findings were more compatible with the renal lesions seen in the generalised Shwartzman reaction. The differences in these two posthulated mechanisms

may have important therapeutic implication for the latter is potentially treatable with heparinisation (Hjort 1965). Recently MacDonald (1970) had shown that heparin could delay the hyperacute rejection of dog kidney allografts. As expected, this form of allograft immunity may also be transferable via the immune sera. Thus incubation of mouse and dog kidneys with the specific antisera could initiate a rejection process which would be continued as 'autologous rejection' when the kidneys were reimplanted into their original hosts (Terasaki 1962, Robertshaw 1969).

Apart from its role in hyperacute rejection, antibody may also be implicated in the late or chronic rejection. Jeannet (1970) found that twelve out of sixteen patients who formed antibody in response to their grafts had poor clinical course as compared to two out of twelve in whom no antibody was detected. Further a high correlation was obtained between the levels of antibody and the presence of obliterative vascular lesions in the grafts.

(iv) Summary

To summarise, both the cell-mediated immunity and humoral antibody production are seen following allografting. The destruction of allografts in most instances is primarily and predominantly an expression of cellular immunity. The 'white reaction' of skin grafts and the hyperacute rejection of kidneys are exceptions, being mediated primarily by humoral antibody.

C) The Biologic Role of the Allograft Reaction

The transplantation of living tissues is an artificial phenomenon, one that Nature surely did not anticipate. Yet we have

already evolved a complex and formidable barrier against it. It has to be assumed that such a process must have some other useful biologic function. Brent (1958) suggested that the allograft reaction is a consequence of the evolution in mammals of an elaborate system of defence against microorganisms. A more charitable viewpoint however was that expressed by Thomas (1959). Briefly, some of our body cells are constantly undergoing mitoses and therefore a certain proportion of mutations is to be expected. Some of these mutated cells may prove to be cancerous and harmful to the body if allowed to propogate. However through the mutations these cells will also acquire a different antigenicity and be recognised as being 'foreign' and hence succumb to the full force of the allograft reaction. Thus functioning as a 'surveillance' force it helps in maintaining the constancy of body genotype. The increased incidence of malignancy in patients with impaired immune process may be viewed as an expression of a poorly functioning 'surveillance' force (Lancet 1969).

III REVIEW OF LITERATURE ON XENOGENEIC TRANSPLANTATION

The idea that a portion of the human body may be of animal origin is a very ancient one as attested by references to such mythological figures as the satyrs, centaurs and chimera. An actual attempt at 'zoografting' was made as early as the seventeenth century by a Russian surgeon who tried to repair a skull defect using the bone chips of a dog. Unfortunately the operation was not looked upon favorably by the Russian Church which ordered the bone chips be removed (Gibson 1955). Although other attempts at cross-specie transplantation were made around the beginning of the century, the modern

study of organ xenografting was started only in the late fifties. Those earlier studies were less a scientific investigation than attempts at satisfying surgical curiosity. In this review the emphasis will be placed on the later works and be restricted to the discussion on transplantation of normal tissues - mainly skin and kidney as examples of primarily non-vascularised and vascularised grafts respectively.

A) Skin Xenografts

(i) Experience with Experimental Animals

The pattern of rejection of embryonic skin seems to be quite different from that of the adult and will thus be discussed separately.

Fate of Adult Skin: Skin of adult animals transplanted onto a xenogeneic host are usually rejected vigorously. Loeb, in 1920, heterotopically transplanted skin in the subcutaneous space among Guinea pigs, dogs, rabbits and pigeons and failed to obtain any growth or evidence of revascularisation of these grafts (Reemtsma 1968). Similarly Egdahl (1958) failed to see any revascularisation of rat-to-rabbit skin grafts; rabbit-to-rat grafts however were consistently revascularised by the third or fourth day and then rejected. Bromberg (1965) working with pig-to-rat grafts noted that even though these grafts appeared 'soft and viable' for three weeks they were never revascularised. Histologically they did not show any cellular infiltration. Pig-to-rabbit grafts however were all revascularised (Bromberg 1965). BenHur (1969) studied the morphologic pattern of rejections in Webster mice of autografts, allografts and xenografts from Guinea pigs, rabbits and rats. He observed that in the first

few days post-grafting, cellular infiltrations, mainly by polymorphs, were seen in all grafts including the autografts. By the third or fourth day the auto- and allografts were revascularised and the cellular infiltration diminished in the autografts but increased in the allografts until they were rejected. All the xenografts from the rabbits and some from the rats were revascularised; however the grafts from the Guinea pigs were not but they were still heavily infiltrated. With the Guinea pigs as hosts, grafts from rabbits, rats and mice were all rejected without being revascularised. This point of revascularisation is central in the discussion of rejection, for if the grafts were not revascularised then they may merely be acting as biological dressings and non-immunological factors as autolytic digestion may be responsible for graft destruction. Alternatively, immune processes may still be implicated and that the lack of vascularisation may be comparable to the white graft reaction of allografts - a manifestation of heightened sensitivity.

'Second set' xenogeneic grafts had been attempted in a few instances. Brautbar (1967) found that second set rabbit-to-rat grafts were rejected in six days as compared to nine with the first set. Further, the rabbit skin transplanted in a rat that had rejected an allograft was rejected faster than a first set implying some crossing-over of antigens between the species. Similar accelerated second set rejection was noted with sheep-to-goat grafts (Perper 1966). Second set pig-to-rat grafts were rejected no faster than the first set; however such grafts were never revascularised and perhaps the hosts were never immunised by the first set grafts (Bromberg 1965). The difficulty in attempting to study second set xenografts is that, with

few exceptions (rabbit-to-rat), the first set xenografts were already rejected in an accelerated fashion.

Fate of Embryonic Skin: Embryonic skin generally seemed to be more readily accepted by the xenogeneic hosts. Green (1953) grafted the embryonic skin of man, cat, rabbit, Guinea pig and mouse into the subcutaneous space of the hamster and DBA mice and showed that about 60% and 40% 'take' in the two respective hosts. Histologically these grafts were free of any cellular infiltration and showed evidence of maturation of their appendages. Further, the rabbit skin maintained in the hamster retained its susceptibility to infection by Shope papilloma virus, a feature not shared by the hamster's skin. Silvetti (1957) grafted bovine skin onto mice, rabbits, rats and dogs and noted that these grafts seemed to adhere to their beds and that their edges were well knitted with that of their hosts. Further, the hosts showed no 'ill effects' from the foreign grafts. Also, second set grafts were rejected more rapidly than the first set suggesting that these grafts were capable of immunising the hosts.

Attempts at Prolonging Skin Xenografts: There are many possible non-immunological factors that may be responsible for the failure of xenografts. The grafts may undergo autolytic digestion, become infected by microorganisms that are innocous to the hosts or the hosts may produce metabolites toxic to the grafts or fail to supply the necessary factors for graft growth (Lance 1968). Nevertheless in those species combination where immunological factors are operable, attempts at prolonging graft survival through interfering with the immune response of the hosts may be fruitful. Table I summarises the attempts made thus far at improving the survival of skin xenografts through immunological manipulations.

Table I

ATTEMPTS AT PROLONGING SKIN XENOGRAFTS

	Donor	Recipient	Prolongation	Author
A.	Steroids:			
	Mouse	Hamster	From 14 to 40 days with 10 mgm. From 14 to 70 days with 30 mgm.	Adams(1958)
B.	Heterologous Antilymphocytic Serum:			
	Rat	Mice	From 21 to 32 days if given pre-op From 21 to 33-38 days if given pre & post-op	Monaco(1966)
	Human Guinea pig Rat Rabbit	Mice	Maintained histologic integrity when examined 3-6 weeks later	
	Goat	Calves	From 7 to 40 days	Donawick(1971)
C.	Heterologous Antithymocyte Serum:			
	Rat	Mice	Survived 'longer' Appeared 'pink'	Chen(1971)
D.	Irradiation of the host:			
	Rat	Mice	Survived for 2 weeks Became vascularised	Lance(1969)
	Pig	Mice	No effect	Bromberg(1965)
E.	Irradiation of the Graft:			
	Pig	Dog	No effect	Rapaport(1970)
F.	Induction of Tolerance:			
	Rat	Mice	ALS plus thymectomy	Lance(1969)
	Chicken Pheasant	Turkey	3 X prolongation	Billingham(1956)
	Goose	Ducks	Some prolongation	Hasek(1959)
	Mice Rabbit	Rats	From 1 to 2 weeks	Egdahl(1958)
G.	Antidonor Antibody ("Enhancement"):			
	Mice	Rats	7 to 19 days	Jeekel(1971)

(ii) Clinical Skin Xenografts

Historically, skin of various mammals, amphibia and birds have all been used to cover skin defects in man (Gibson 1955). Most of the recent observations on clinical skin xenografts came from clinical trials in which animal skins were being used as temporary biological dressings. Thus skin of bovine embryoes and calf were noted to survive as grafts for over two weeks (Rogers 1958, Silvetti 1957). Histologically, intact dermis was noted as late as the fourteenth post-operative day with the bovine embryo skin. Further there was very little adverse reaction in the host from the presence of these animal skin. Both canine and porcine skin had been used as temporary dressings in cases of severe burns. Bromberg (1965) in using pig skin noted that, clinically, the xenografts were just as effective as the allograft in providing a temporary dressing. Interestingly, in one of the patients who received a 'second set' xenograft, the graft was sloughed off within 48 hours. This could mean accelerated rejection or, of course, technical failure. Switzer (1966) noted that those canine skin grafts that were left for over five days tended to adhere to the underlying beds, but no observations were made regarding whether there were any vascular connections between the graft and host. Rapaport (1970), using porcine skin to cover burn areas, reported impressive clinical improvements in his patients - relief from pain, decreased incidence of sepsis and overall general improvement. However in this study no attempt was made to evaluate the fate of the grafts.

It is doubtful that animal skin will ever be used as an actual graft with a view of permanence. The large surface area of

the body from where repeated autografts can be taken, the remarkable ability of skin wounds to contract and re-epithiase and the dangers inherent with immunosupression all tend to limit the use of both allografts and xenografts in a clinical setting. However as temporary biological dressings, animal skin may have a definite role to play - they are easily available in large quantity, provide good coverage of the wound thereby reducing pain, fluid loss and wound contamination and they seem to stimulate the granulation of the underlying bed (Rapaport 1970).

B) Renal Xenografts

(i) Experience with Laboratory Animals

With the development of a successful surgical technique for blood vessel anastomosis early in this century, there followed a rapid application of this technique to the transplanting of vascular organs - both allografts and xenografts (Reemtsma 1968). Although the observations of these early experimenters were limited to gross examination of the grafts they must had realised that the barrier against successful cross-species transplantation, like that of intra species, is immunological rather than technical for no further experimentations were attempted after 1926 till the fifties.

An attempt at systematically studying the phenomenon of cross-species rejection was first made by Brull in 1956 (Brull 1956). Having devised a technique for studying renal blood flow and function, Brull perfused ex vivo, a goat's kidney with sheep's blood and a cat's kidney with a dog. He observed that renal blood flow rapidly declined within a few minutes after perfusion and since on examination these

grafts were free of blood clots, he attributed the decline in blood flow to vasoconstriction. Mowbray in 1961 extended this observation when he found that the rejection of a rabbit kidney by a dog could be delayed by prior treatment of the host with an anti-serotonin drug, an observation that could not be duplicated by later workers (Marceau 1965). Brull however advanced the experimental technique significantly when he showed that the in vivo findings could be reproduced in vitro by perfusing the rabbit kidney with whole dog's blood. In this way the phenomenon could be further studied in vitro by manipulating the composition of the perfusate, a technique much utilised by later workers. Brull himself showed that the decline in blood flow was obtained only with using those perfusates that contained the gamma globulin fraction, thereby implicating for the first time a primary role for the antibody. Way (1965) in studying renal transplantation between members of various species found that grafts between members of the same order survived for 7 - 12 days while those between orders were rejected more rapidly and violently. The dog however was exceptional in that it was a particularly hostile host to any xenograft, rejecting it within minutes. This 'hyperacute rejection' of the xenograft was further studied by Perper (1966) using the pig-to-dog model and showed that the classical immunosupressants - steroids, azathioprine and Actinomycin C - did not alter the course of the rejection. Further, the native serum of the dog was cytotoxic to pig kidney cells suspension and suggested that the hyperacute rejection was initiated by the preformed antibody. Since then, other workers have pointed out to the remarkable similarity, functionally and morphologically, between the hyperacute rejection of xenograft

and allograft - in both the destruction is dramatic and rapid and accompanied by the trapping of formed blood elements, complement and antibodies by the graft, changes in the coagulation factors and the formation of microthrombi in the graft vasculature (Boehmig 1971, Rosenberg 1969). Histologically the graft interstitium is remarkably free of cellular infiltration and the histologic changes were confined mainly to the blood vessels and glomeruli. Further by interfering with any of these factors the hyperacute rejection process could be delayed albeit only briefly. Table II summarises the current experimental attempts at prolonging renal xenograft survival.

(ii) Clinical Renal Xenografts

Attempts at clinical renal xenografting were made at about the same time as the early experimental work. These early trials, like their experimental counterpart, were uniformly unsuccessful. It was certainly premature at that time to attempt xenogeneic transplantation when the mechanism underlying the rejection of allografts had not been fully elucidated as yet.

The modern scientific study of clinical renal xenografting was made mainly at two centers - Reemtsma's group at Tulane and Starzl's at Denver (Reemtsma 1964, Starzl 1964). The Tulane group used chimpanzees as the donors and the Denver group, baboons. The chimpanzee was chosen because of its close taxonomic relationship to man and also it shares common blood groups and similarity in renal functions with that of humans. In Reemtsma's six cases, good immediate post-operative renal function were noted in all except one. This was a woman who received a kidney from a chimpanzee with a different blood group. The triple combination of immunosuppressants - azathioprone, steroid

Table II

ATTEMPTS AT DELAYING HYPERACUTE REJECTION OF RENAL XENOGRAFTS

A. DIRECTED TOWARDS THE HOST:

i) Decreasing the level of preformed antibody - absorption (Giles 1970), selective plasmapharesis (Merkel 1970).

ii) Decreasing the complement level - e.g. Cobra venom (Gewurz 1967).

iii) Decreasing the level of formed blood elements - e.g. platelet (Rattazzi 1970).

iv) Interfering with the coagulation process - e.g. citrate (Kun 1971).

v) Inhibiting the release of vasoactive compounds - e.g. Trasylol (Ghilchik 1971).

vi) Induction of tolerance to graft (Owens 1968).

B. DIRECTED TOWARDS THE GRAFT:

i) Antigenic "masking" - use of telopeptide-poor collagen (TPC) (Ellis 1969).

and Actinomycin C - together with graft irradiation were used. Most of the xenografts failed within a couple of months; one however survived for nine months. The cause of death in all instances was sepsis, a complication of the heavy immunosuppressive regime. Most interestingly, some instances of threatened acute rejection were successfully reversed by increasing the immunosuppressants. Pathologically apart from the acute tubular necrosis, edema and punctate hemorrhages the kidneys were remarkably free from heavy cellular infiltrations. With the baboon kidneys, the results were less encouraging. The rejection crises were more frequent and less easily reversed. Pathologically the kidneys appeared more seriously damaged. They were enlarged and edematous with areas of blotchiness and petechial hemorrhages. Histologically they showed heavy cellular infiltrations. Apart from the chimpanzees and the baboons, the Rhesus monkey had been used as a donor in one instance (Reemstma 1968).

C) Host Response to Xenografts

In discussing the xenograft reaction separate from that of the allograft, it may be implied that the two are quite different. In fact judging from the results of the early experimenters this may not be an invalid conclusion. Thus allografts were usually rejected after a variable period of normal function whereas xenografts were rejected more dramatically and almost immediately. Further, while the rejection of allografts could be delayed by the classical immunosuppressants, that of xenografts could not (Perper 1966). It is known that this distinction between the two is not quite clear cut.

Thus the hyperacute rejection of kidneys are seen both in allografts and xenografts and that the usual immunosuppressants could delay the rejection of some xenografts (Perper 1966 - see Table I). The current view is that the rejections of both allograft and xenograft are qualitatively similar processes being immunologically-mediated and having a similar overlapping spectrum of reactivity ranging from the slow cell-mediated immunity at one end to the violent and dramatic antibody-mediated immunity at the other (Reemtsma 1971).

The antibodies that figure prominently in the rejection of xenografts, unlike that of the allograft situation, are for most instances preformed and naturally occurring, that is, not in response to any known immunisation. There are two possibilities as to the origin of this 'naturally occurring' antibodies - one is that they are 'natural' in the same sense as for example the anti-A or anti-B isoagglutinins. Two, that these antibodies are produced in response to immunisation with natural antigens (for example, microorganisms) some of which cross-react with the xenoantigens. The latter is exemplified by the heterophile (Forssman) antigen which is found on the red cells of many species is also present in various bacteria (Humphrey 1970). Reemtsma (1971) has constructed a theoretical model which showed that the wider the phylogenetic relationship between the species involved the more likely is for this cross-immunisation to occur. It would be interesting to trace the development of these antibodies ontogenically in normal and gnotobiotic animals. As to the nature of these antibodies, it is known that the native sera of certain animals, especially the dog, have hemolytic, various agglutinating and cytotoxic titers against a variety of xenogeneic target cells (Perper 1966).

Xenotransplants do evoke antibody response and these can be easily demonstrated both *in vivo* and *in vitro* (Woodruff 1960). Thus rabbits immunised with tissues from various animals produced antisera that may be either predominantly species-specific or organ specific depending upon the species and tissue involved. Thus an antibody developed to a kidney of a particular species would cross-react with the kidneys of other species (organ specific) whereas the anti-brain antibody would react with other tissues of the same donor but not with the brain of other species (Species-specific) (Woodruff 1960). Sachs (1971) showed that when mice, rats and rabbits were immunised with xenogeneic lymphocytes of rats and mice of different strains the antibody developed was not only species-specific but also strain specific. The strain specificity was observed only when the species-specific component of the antisera had been appropriately absorbed.

What is the role of cell-mediated immunity in xenotransplants? In xenografts between closely related species, this is the predominant reaction (Perper 1966). In transplants between widely disparate species the role of CMI cannot be easily assessed *in vivo* due to the overwhelming effect of antibody-mediated rejection. Using the mixed lymphocyte culture technique, a good *in vitro* correlate of cellular immunity, it had been shown that the degree of blastic transformation when allogeneic lymphocytes were cultured together was directly proportional to the degree of genetic disparity between the two (Gordon 1972). However when lymphocytes from different species were cultured together, the degree of blastic transformation was less than if the lymphocytes had been cultured with allogeneic cells despite the obvious wider genetic disparity in the former case (Wilson 1970,

Hersh 1971). This has led to the suggestion that in the circulating pool of lymphocytes we have more cells that are capable of recognising alloantigens than xenoantigens and that during evolutionary process we have selected these alloantigen-recognition cells in favor of the other cells. This is in contrast to the views of Reemstma (1971) mentioned earlier in that the various species are already immunised against each other through the mechanism of cross-reactivity of natural antigens. However this mixed lymphocyte culture technique has not been widely used to investigate other species-combination especially between closely related species.

Immunological considerations aside, there may be other possible factors that may govern the long term survival of xenografts. The known physiological and biochemical differences in function of the same organ in various species and the subtle differences in the milieu interieur of the various hosts of different species may prove to be a more significant barrier to the long term survival of xeno-grafts (Lance 1968).

D) Rationale for Studying Xenogeneic Transplantation

At present, one of the limiting factors preventing the wider clinical application of renal transplantation is the inadequate supply of suitable donor organs. The need to find suitable non-human donors will be felt more and more. Such non-human organs may be used either for permanent replacement, as with a kidney transplant, or to provide temporary metabolic support as in the management of hepatic coma. Thus the wider study of cross-species transplantation is not without clinical relevance.

The question of relevance aside, there is an intrinsic value in studying the phenomenon of cross-species rejection. Following the thesis that the intensity of rejection is a function of the genetic disparity between the donor and recipient, the assessment of the rejection phenomenon provides another gauge for determining the taxonomic relationship between species. Information obtained from such studies may, in addition to those obtained from comparative anatomy and physiology, help clarify the phylogenetic relationship between various species.

IV ENHANCEMENT, BLOCKING ANTIBODY AND ANTIBODY FRAGMENTS

Earlier it was alluded that the presence of alloantibody directed against the grafts can have the effect of prolonging the survival of these grafts, a phenomenon of 'immunological enhancement'. The earliest observation of this phenomenon was attributed to Flexner who in 1907 showed that the prior inoculation of killed sarcoma cells in rats would favorably influence the survival of subsequently injected live sarcoma cells (active enhancement) (Russel 1971). In 1958, Kaliss extended the observation when he showed that the phenomenon was specific for the tumor used to immunise the host and that it was readily transferable to syngeneic hosts via the immune serum (passive enhancement) (Kaliss 1966). There are two possible explanations for this phenomenon - one, the antisera somehow induces physiological changes in the tumor cells enabling them to survive better in the host, or two, that the antibody may interfere with the immune response of the host to the graft (Uhr 1968).

If the immune response can be conceptualised as consisting

of three phases - an afferent, central and efferent phase, then one can postulate three possible sites of action for the antibody. Firstly, the antibody may neutralise the antigen thereby rendering it less immunogeneic or completely neutralising it (afferent inhibition). 'Central inhibition' would involve the interference with the interaction of the antigen with the immunocompetent cells rendering the latter less reactive. Lastly the antibody may bind the antigenic sites on the grafts without destroying them and protecting them from subsequent attack by the various effector mechanisms of the immune response - that is, as a 'blocking antibody'. There are extensive evidences to support each of these mechanisms and these have been extensively reviewed by Kaliss (1966), Uhr (1968) and Snell (1970). The present discussion will be an extraction from these sources.

Using the appropriate assay system, it had been shown that passively-administered antisera to an antigen could depress both the humoral and cellular responses to that antigen (Uhr 1968, Snell 1970). Moller (1965) showed that in mice, the passive administration of antibody to sheep erythrocytes before antigenic stimulations would suppress the appearance of splenic antibody-forming cells. Antibody given after antigenic stimulation, however, was less effective. Using the salmonella flagellin antigen, Diener (1971) showed that mouse spleen cells, preincubated with a particular ratio of the antigen and the specific antibody would, when subsequently exposed to immunogenic doses of the same antigen <u>in vitro</u> and <u>in vivo</u> developed a state of depressed immunological responsiveness. McKenzie (1971) working with rat skin allografts, showed that the hosts receiving the antidonor antisera had fewer blast cells, germinal centers and

plasma cells in the lymph nodes draining the graft site as compared to non-treated hosts. Further these nodal lymphocytes showed less reactivity when tested eight days later with target tumor cells.

The evidences for a peripheral action for the antibody came from both *in vivo* and *in vitro* studies. Moller (1964) incubated allogeneic tumor cells with their alloantibody and then implanted them in one thigh of a mice; and on the other thigh he implanted the untreated tumor cells. The untreated tumor cells were rejected while the treated ones grew, indicating that the level of action of the antibody is at the graft rather than the host. Equally convincing are the *in vitro* studies. In rabbits, the Shope virus-induced papilloma may either regress or persist, developing into a carcinoma. The lymphocytes of both the 'regressors' and 'persistors' were equally inhibitory to the growth of the papilloma *in vitro*. However if the target tumor cells were first incubated with the sera of the 'persistors' and then mixed in culture with the lymphocytes of either rabbits, no inhibition of growth occurred. Incubation of the target cells with the serum of the 'regressor' rabbits or incubating the lymphocytes of either rabbits with either of the two sera did not prevent any inhibition. Thus the sera of the 'persistor' rabbits seem to act on the tumor target cells and blocking the action of the immune lymphocytes and this probably explains why the Shope papilloma survive in these rabbits (Hellstrom 1970). Similar 'blocking' activity have been detected by Hellstrom in the sera of patients with various disseminated malignancies. Wegmann (1971) working with tetraparental mice (formed by fusing the embryos from parents of different histocompatibilities) showed that the parental fibro-

blasts were destroyed in vitro by the lymphocytes of these chimera. However if the culture conditions were performed in the presence of the sera of the tetraparental mice this destruction was prevented. Thus it seemed that these tetraparental mice have 'forbidden clones' of lymphocytes capable of recognising 'self' antigens but are prevented from carrying out self destruction by the presence of factors in the serum. Further studies from Hellstrom's laboratories have indicated that these blocking factors are antigen-antibody complexes rather than antibody (Hellstrom 1970). This ties in with Diener's observation on requiring exposure to both antigen and antibody to effect immune suppression (Diener 1971). Although such formal separation between 'central' and 'peripheral' effects of antibody may be readily demonstrated with the various assay systems, it is conceivable that in the actual in vivo phenomenon, both factors may be operative, depending on the concentrations and nature of the antigen and antibody and the phase of the immune response when such factors are introduced.

This phenomenon of enhancement has been observed with a wide variety of normal and neoplastic tissue grafts. Enhancement of normal tissue grafts have been achieved with ovaries, skin and kidneys. Of these the most successful is the kidney (Batchelor 1969, Stuart 1968) and least the skin allografts (Zimmerman 1969, Cohen 1971). One of the difficulties with attempts at enhancement is that isoantibodies to all the alloantigens on the graft must be present in the system; if not those antigens not 'covered' will be able to evoke and be susceptible to the usual allograft response of the host (Russel 1971). Cohen (1971) attempted to raise such an antisera to rat skin allografts by immunising the host through repeated (over

ten times) skin grafts from the prospective donors, but the degree of enhancement obtained was not better than that achieved by other workers. With skin xenografts, the result with passive enhancement is surprisingly impressive. Jeekel (1971) was able to prolong up to 38 days the survival of mouse-to-rat skin grafts by giving the hosts specific antisera. The other possibility is to combine immunosuppression and passive enhancement, the rationale being to suppress the host's response to the 'uncovered' antigens. Judging from the results of McKenzie's work (1971) such combinations were indeed quite superior than either alone in prolonging the survival of skin allografts in rats.

An innovative approach at passive enhancement was provided by Nelken (1968). His work is interesting in two respects - first the antisera were heterologous and second they were used to treat the graft rather than the host. Antisera to skin of Hebrew white and Lister rats were raised in chicken and rabbits through repeated injections of epidermal cells. The rat skin graft was then incubated with the antisera for 20 hours at 6°C and then allografted. By this treatment he was able to prolong the graft survival for a few extra days. The suggested hypothesis was that the antibody had combined with the antigenic sites on the grafts rendering them either less immunogenic or, protecting them from the host's immune response.

In both normal and tumor tissue grafts, antibody can have either a protective (enhancing) or cytotoxic (destructive) effect and that the final outcome in each case will depend on the balance between the two (Russel 1971). Clearly if one can favour the balance one way or the other one can advantageously influente the outcome of a particular graft system - that is enhancement in case of normal tissue grafts and destruction with neoplastic grafts. One approach

to this would be to assume that the enhancing sera to be different from the cytotoxic one. Attempts can then be made at characterising their physico-chemical properties in sufficient details as to facilitate their separation from the non-enhancing antibodies. Takasuzi (1969) had shown that, in mice, enhancing activity for Sarcoma I tumor grafts was detected only in the $I_gG\gamma_2$ fraction. The second approach would be to separate within the antibody molecule itself the antigen-binding from the cytotoxic component, and then using the appropriate antibody fragment.

The antibody molecule is made up of two identical heavy (H) chains and two identical light (L) chains joined together by di-sulphide and various physical bonds. The antigen-binding capacity of the molecule resides at one end of the H chains. Digestion with pepsin results in the degradation of the molecule into two components - the divalent $F(ab)_2$ fragment consisting of portions of the pair of H chains and all of the L chains, and an Fc fragment made up of the portions of the H chains only. The antigen-binding properties are retained in the $F(ab)_2$ fragment. The portion of the antibody molecule giving rise to the Fc fragment is responsible, in the intact molecule, for most of the biologic properties of the antibody, in particular, the ability to fix complement. Thus the $F(ab)_2$ fragment, while still retaining its antigen-binding capacity, is unable to bind complement and hence non-cytotoxic (Nisonoff 1971). The $F(ab)_2$ fragment, like the intact molecule, could suppress the humoral response to particulate antigens (Uhr 1968). Of interest here is the ability of the $F(ab)_2$ fragment to bind cell-surface antigens and, being non-cytotoxic, will act as a 'blocking' antibody covering up the antigenic sites and

protecting them from the 'effectors' of the host's immune response. Thus Broder (1968) showed that the antisera to mouse Ehrlich Ascites tumor cells (EAT) were cytotoxic to the target tumor cells in vitro while the pepsin-digested antisera were not. Further, EAT cells incubated with the digested antisera would survive when implanted in the mouse while tumor cells incubated with the native antisera did not. Turning to normal tissue grafts, Shaipanich (1971) was able to markedly prolong the survival of (Lewis X Brown Norway) Fi hybrid to Lewis renal allografts by prior treatment of the grafts with the pepsin-degraded antisera. The prolongation was even more impressive when graft pre-treatment was combined with systemic administration in the host with the $F(ab)_2$ fragment. Treatment of the grafts with the intact antisera did not prolong their survival. The systemic administration of the native antisera however, resulted in prolonged survival of the grafts though not to the degree obtained with when the grafts were pre-treated with the degraded antibody. A similar prolongation of renal allografts in the rats with the systemic administration of the intact antisera was obtained earlier by Stuart (1968) and Batchelor (1969). In monkeys, prior perfusion of the kidneys with the pepsin-degraded antisera (raised through repeated skin grafts) was effective in delaying the hyperacute rejection of these kidneys (Kobayashi 1971). Most interestingly, the $F(ab)_2$ of the antisera raised in a third party (that is not the prospective recipient) was equally effective as the antisera raised in the prospective recipient. The use of a third party to raise the antisera had been used clinically earlier by Batchelor (1970). In this case the recipient after receiving a kidney from her mother, was treated with the enzyme-

degraded antisera against the donor (mother) tissue. The antisera used was raised in the father using the mother's lymphocytes as antigens.

In another rejection situation, if a kidney was perfused with the hyperimmune sera against that kidney and then re-implanted into its original host, it would undergo rejection, an'autologous rejection' phenomenon (Robertshaw 1969). However if the kidney was first perfused with the $F(ab)_2$ of the immune sera before incubation with the intact antisera and then re-implanted, no autologous rejection occurred (Smith 1971). Thus the antibody fragment of the immune sera acted as blocking antibody and protecting the grafts from subsequent attack by either antibody or immune cells.

Thus far we have seen the antibody fragments being used as blocking antibody in protecting neoplastic tissue xenografts and normal tissue allografts. In this study an attempt is made to extend this observation to normal skin and renal xenografts.

V PURPOSE OF THE PRESENT STUDY

In the preceding discussion, it was noted that with certain transplant models, the prior treatment of the grafts with the specific antidonor $F(ab)_2$ fragments resulted in their delayed rejections. With allografts, this was seen in the Fi-to-P renal transplants in rats and in the hyperacute rejection of monkeys' and dogs' kidneys. With xenografts, this was demonstrated in the mouse Ehrlichs Ascites tumor cell transplants in Guinea pigs.

In this study, an attempt is made at extending these observations to normal tissue xenografts. For this, the following

transplant models were chosen:

A. Rabbit-to-dog renal xenografts

B. C57BL/6 mouse to Lewis rat skin xenografts.

The kidney and skin grafts were used as examples of a primarily vascularised and non-vascularised organs respectively. The rabbit-to-dog renal transplants are rejected within minutes, being primarily mediated by the preformed antibodies. It is thus a convenient and ready made model for hyperacute rejection. Mouse-to-rat skin transplants are rejected without their being revascularised, a reaction comparable to the 'white graft' rejection of allografts - a reflection of heightened sensitivity.

The general scheme of the experiments was as follows: The grafts were incubated with the specific antidonor $F(ab)_2$ prior to being transplanted and their survivals compared to those similarly treated with the intact antidonor antisera, control antisera and their $F(ab)_2$ and saline. Homologous antisera were used for the kidneys and isologous antisera for the skin.

VI METHODS AND MATERIALS

A) Rabbit-to-Dog Renal Xenografts

Donors: Adult New Zealand White Rabbits

Recipients: Adult Mongrel Dogs

Figure I outlines the general scheme of the experiment. Preparation of antidonor antisera: 1-2 X 10^8 lymphocytes of the prospective donor rabbit (obtained at laparatomy from the mesenteric nodes) were injected intramuscularly, with complete Freund's adjuvant, weekly, into the non-recipient dog. The first injection was with fresh cells, subsequent ones were with frozen cells. A week after

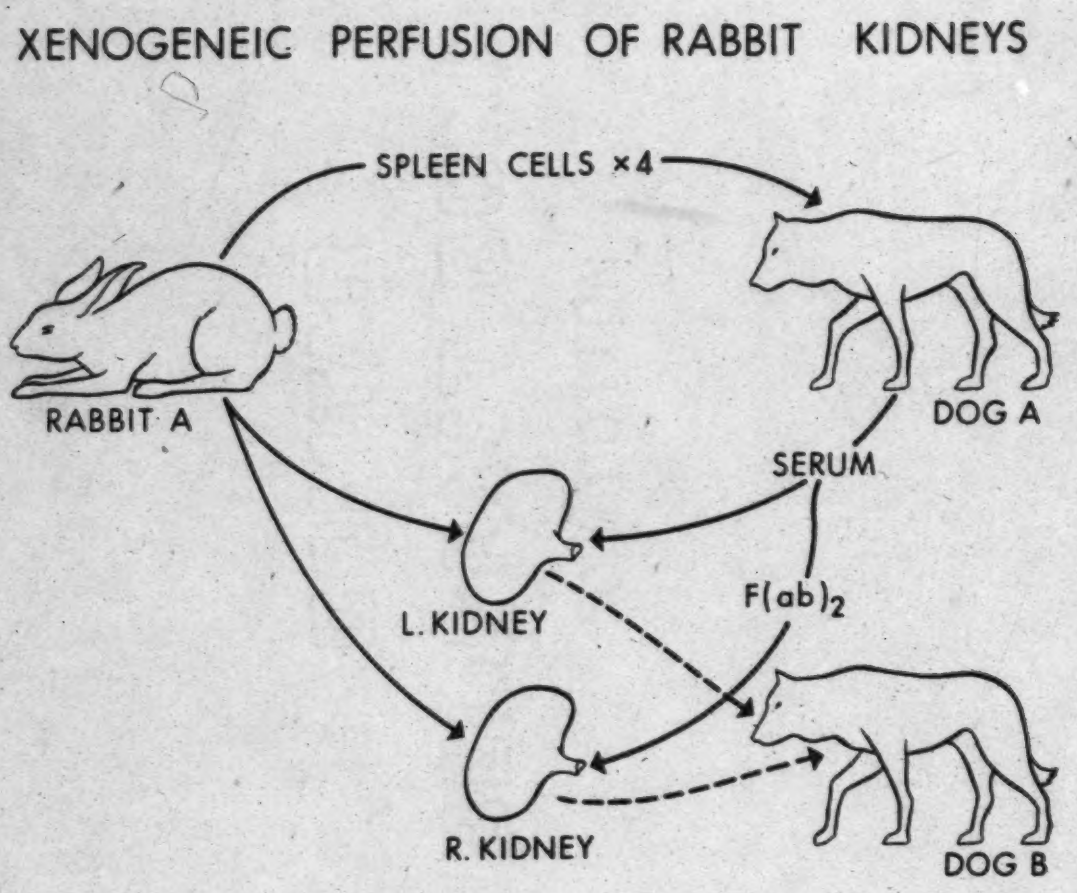


Fig. 1 Scheme of experiment with renal xenografts

the fourth injection, about 300 c.c. of blood was collected and half of the antisera obtained was subjected to pepsin-digestion, the other half was stored frozen till used. One kidney of the donor rabbit was incubated with the intact antisera and the other with $F(ab)_2$ prior to being perfused by the recipient, non-immunised dog.

Xenogeneic perfusion: The donor rabbits were re-operated (using halothane inhalation anesthesia) and their renal vessels cannulated. The kidneys were flushed with saline until the venous effluent was clear and then perfused with the specific antidonor antisera (or $F(ab)_2$) for 25-30 minutes using a pulsatile flow recirculating Harvard pump and maintaining a mean perfusion pressure of about 80 m.m. of mercury. After incubation, the kidneys were again flushed with saline and were now ready for xenogeneic perfusion.

For the control groups, the kidneys were incubated with the control antisera or their $F(ab)_2$ (obtained from dogs recently immunised for distemper) or saline. All the perfusates contained 2 i.u. heparin and 50 mgm. of procaine HCl per ml.

The recipient dog was anesthetised with intravenous Nembutal (30 mgm/Kgm) and received 300 i.u./kg. of heparin prior to cannulating both their femoral arteries. One cannulae led to the pressure transducer, the other to be connected to the recipient rabbit's renal artery. The dog's arterial pressure was maintained throughout the experiment with saline infusion. During xenogeneic perfusion, the minute graft blood flow was measured by collecting the venous outflow in a graduated cylinder and the arterio-venous gradients of formed blood elements determined at frequent intervals. End point rejection was when the graft blood suddenly decreased or

became less than 10% of the initial flow.

B) Mouse-To-Rat Skin Xenograft (Figure 2)

Donors: Male C57BL/6 mice

Recipients: Lewis rats

Technique: Full-thickness donor skin, measuring 2 cm^2, was taken from the abdomen of the mouse and grafted onto the postero-lateral aspect of the chest using the standard technique (Billingham 1961). A control autograft was done on the opposite side. The grafts were covered with sofratoule dressing and enclosed in a rib-cage cast for three days. After the fourth day, the animals were anesthetised with Penthrane and the graft sites examined daily under the stereomicroscope by an independent observer. End point of rejection was when the grafts were hard and necrotic.

Experimental Groups: Prior to grafting, the grafts were incubated at room temperature for 4-6 hours with the specific antidonor antibody (Group D), or its $F(ab)_2$ (Group E), control antisera (Group B) or their $F(ab)_2$ (Group C) or saline (Group A).

Preparation of antidonor antisera: Eighteen Lewis rats were injected intraperitoneally with 2 X 10^7 C57BL/6 mouse spleen cells, in complete Freunds Adjuvants, weekly for three weeks, followed by weekly injections (thrice) of homogenised epidermal cells. A week after the last injection, the rats were exsanguinated and their sera collected and pooled. Half of the antisera were subjected to pepsin degradation.

C) Pepsin Digestion: The rat's immunoglobulin was purified

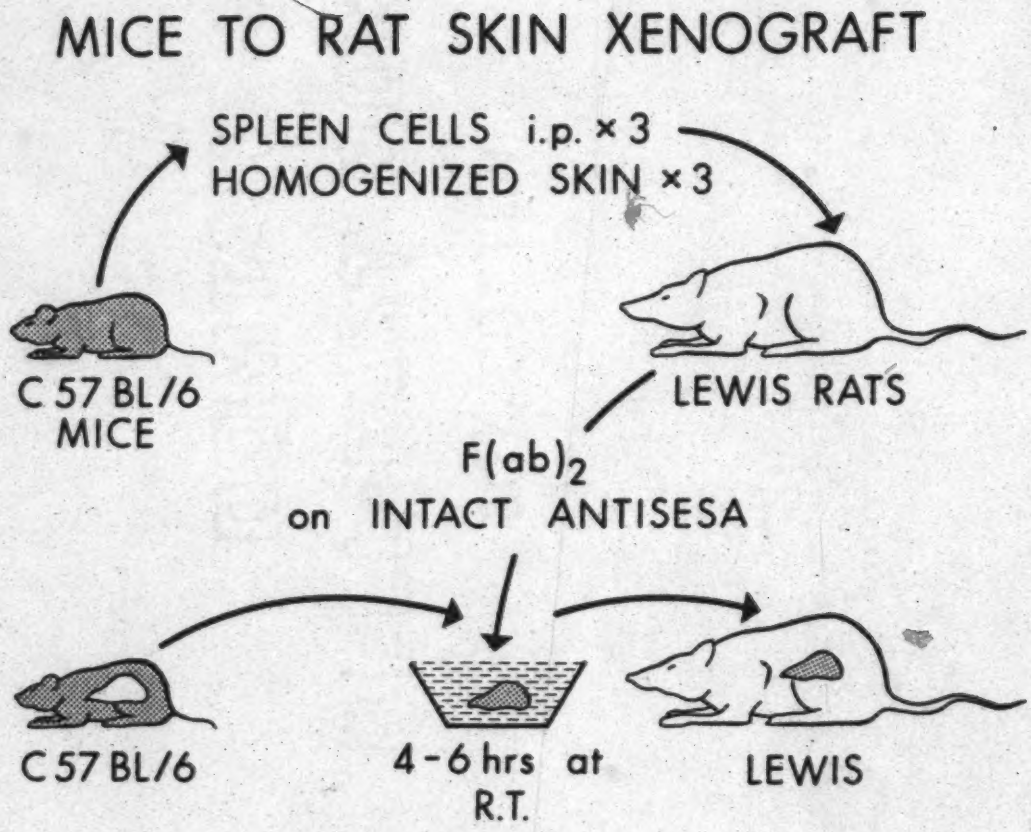


Fig. 2 Scheme of experiment with skin xenograft

by precipitation with saturated ammonium sulphate solution; the dog's with repeated incubation with DAE Sephadex 50. Pepsin digestion was carried out at pH 4.0 at 37°C for 72 hours for the rat antibody and 36 hours for the dog antibody using a pepsin globulin ratio of 1:50 (Williams 1967). The final products were dialysed overnight against buffered saline (pH 7.4) and stored at -20°C.

The sedimentation coefficient of the dog immunoglobulin was 5.9; the $F(ab)_2$ 3.0 (Figure 3). The lymphocytotoxic titers of the dogs' antidonor sera (ADS) ranged from $1/10^2$ - $1/10^4$. Native dog-sera has a titer of 1/8. The hemagglutinating titers of the dogs' ADS were from 1/265 to 1/1024; the $F(ab)_2$ 1/4 - 1/16; the native sera has a titer of 1/4. The hemeagglutinating titer of the rat ADS was 1/1024; the $F(ab)_2$ 1/2. Native rat sera has no agglutinating activity.

VII RESULTS

A) Rabbit-to-Dog Renal Xenograft

Two of the six dogs immunised with the rabbit cells developed severe abscesses and had to be killed before completion of the immunisation schedule. Thus antisera to only four donor rabbits were obtained for the experimental groups D and E.

Table III shows the rejection times of the xenografts in the various experimental groups. Pretreatment of the grafts with the specific anti-donor $F(ab)_2$ or intact antisera, did not delay their rejections. All the kidneys in all the groups were rejected around ten minutes. Further, the specific $F(ab)_2$-treated kidneys were just as effective as the control kidneys in trapping formed blood elements - platelets and leucocytes (Figure 4).

Fig. 3 Ultracentrifuge pattern of dog immunoglobulin (upper) and $F(ab)_2$ (below). The sedimentation coefficient of the intact antibody is 5.9; the $F(ab)_2$ 3.0.

Table III

REJECTION TIMES OF XENOGENEICALLY PERFUSED RABBIT KIDNEYS AFTER VARIOUS PRETREATMENT

Group	Graft Pretreatment	No. of Kidneys	Rejection time in minutes	S.D.
A	Saline	6	10.5	0.6
B	Control Antisera	6	9.0	2.9
C	Control $F(ab)_2$	5	8.8	2.5
D	Antidonor Antisera	4	6.8	1.7
E	Antidonor $F(ab)_2$	4	6.3	2.2

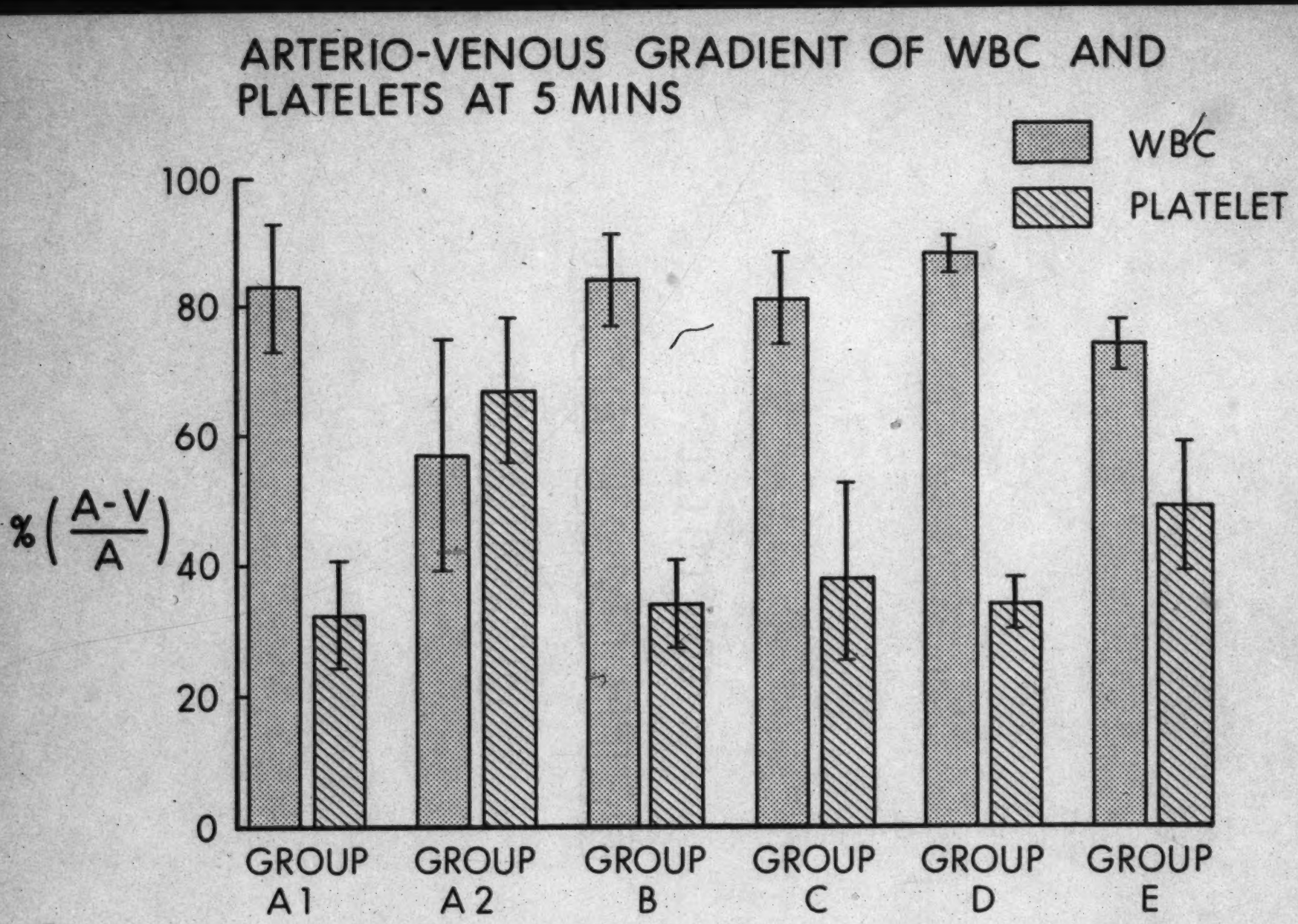


Fig. 4 Arterio-venous gradient of leucocytes and platelets at five minutes after perfusion

Within minutes of establishing blood flow, the kidneys rapidly became pink and, in a few instances, began secreting drops of urine. The blood flow usually peaked in about 3 - 6 minutes. Thereafter, coinciding with the rapid decline in the flow (Figure 5), the kidneys appeared mottled and congested, with areas of cyanosis and petechial hemorrhages (Figure 6). When biopsied at this time, they bled very little, oozing dark blood from the biopsy site. On sectioning the renal vessels and their main branches were free of clots. Histologically, these kidneys showed diffused interstitial hemorrhages and swelling of the tubular cells. Their glomeruli and small arterioles showed infiltration with diffused, granular and eosinophilic staining material (Figure 7).

B) Mouse-to-Rat Skin Xenografts

The mean survival times (MST) of the skin xenografts in the control groups were: Group A (saline-treated) 5.93 ($\pm$.83) days; Group B (control antisera-treated) 5.69 ($\pm$.85) days; Group C (control $F(ab)_2$-treated) 5.67 ($\pm$.78) days. The MST for the experimental groups D (specific antisera-treated) and E (specific $F(ab)_2$-treated) were 7.36 ($\pm$1.43) and 7.22 ($\pm$0.97) days, respectively (see Table IV and Figure 8).

Pretreatment of the skin xenografts with specific anti-donor antibody or its $F(ab)_2$ fragments significantly prolongs the survival of such grafts (for Groups A:E, $p < 0.005$, Groups A:D, $p < 0.005$). There was no difference between the MST of the specific antisera and $F(ab)_2$-treated groups - both were equally effective.

Seven out of the eleven specific antisera-treated grafts

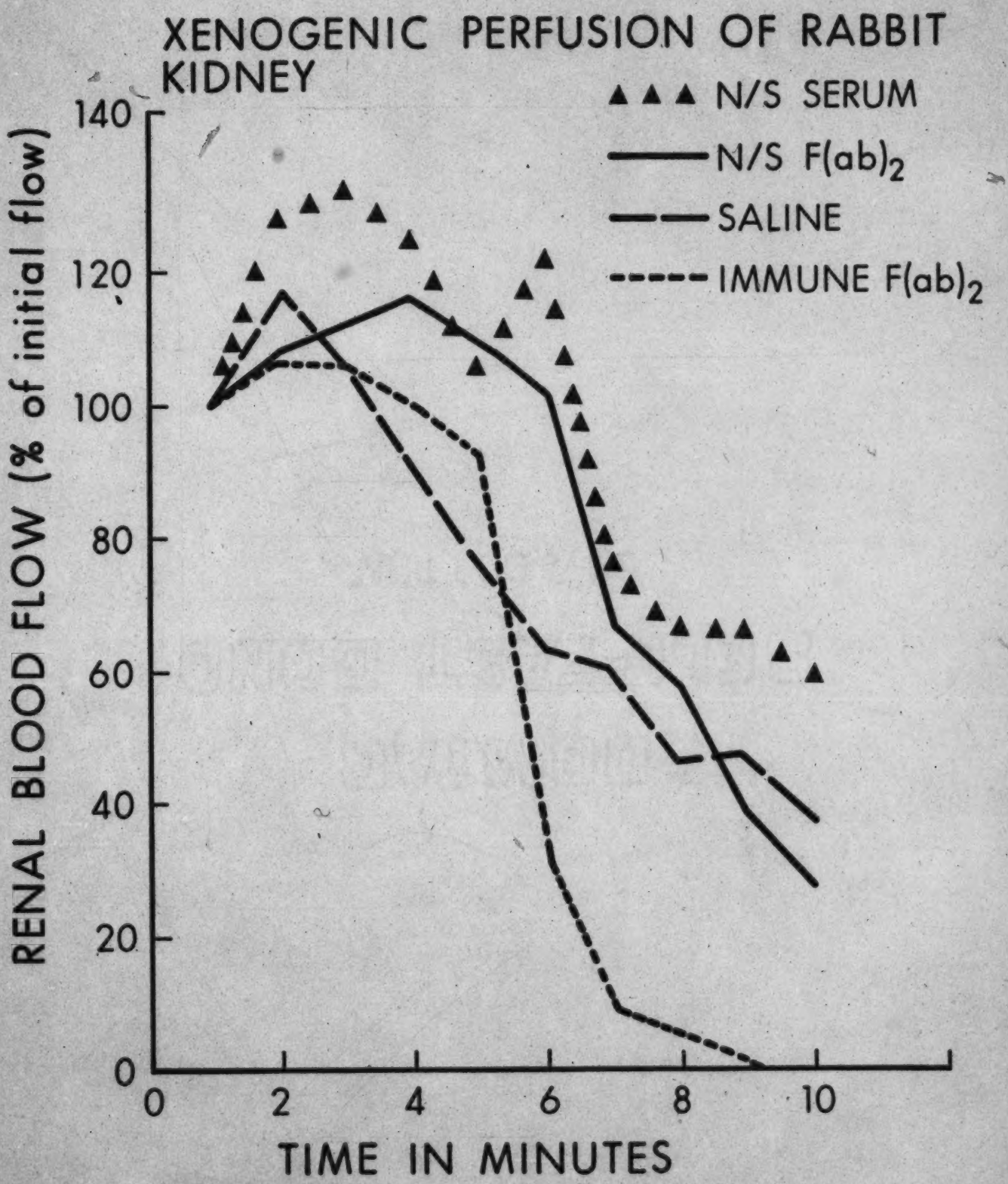


Fig. 5 Pattern of renal blood flow of renal xenograft

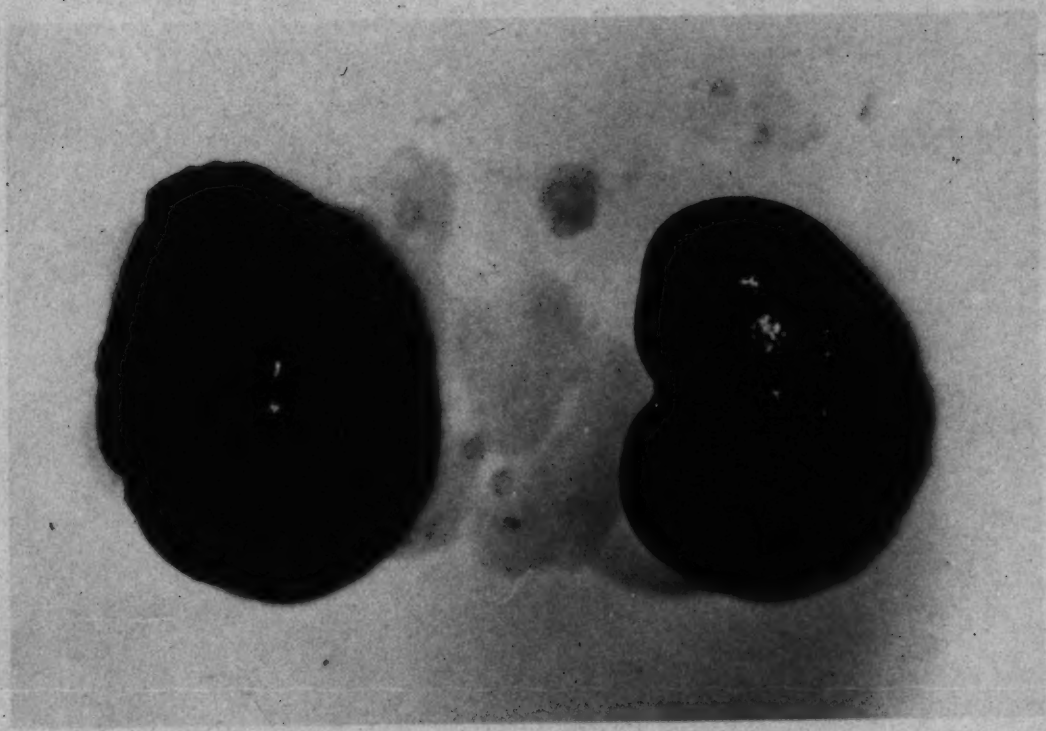


Fig. 6 Gross features of hyperacutely rejected renal xenograft

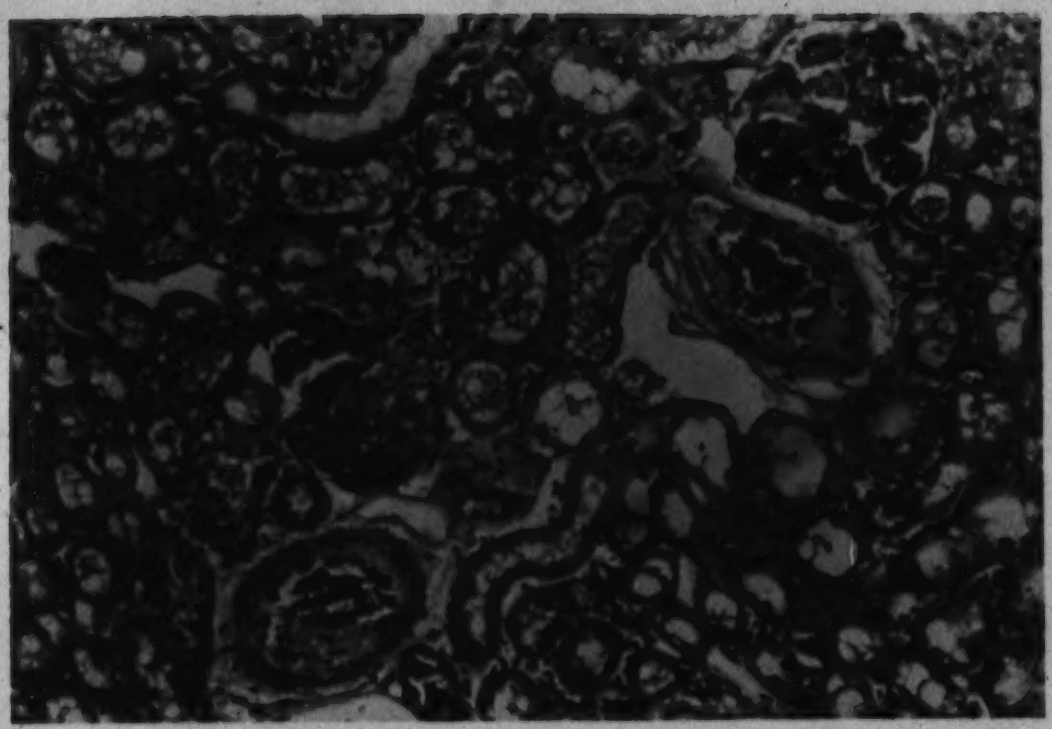


Fig. 7 Histologic features of hyperacutely rejected kidneys. Note the infiltration of glomeruli and arteriole and swelling of tubular cells.

Table IV

REJECTION OF MOUSE TO RAT SKIN XENOGRAFTS

Experimental Group	Days Survived	No.
Group A		
(saline-treated)	5	5
N = 14	6	5
MST = 5.93 (± 0.83) days	7	4
Group B		
(Non-specific sera-treated)	5	7
N = 13	6	3
MST = 5.69 (± 0.85) days	7	3
Group C		
(Non-specific F(ab)$_2$-treated)	5	6
N = 12	6	4
MST = 5.67 (± 0.78) days	7	2
Group D		
(Specific antisera-treated)	5	1
N = 11	6	2
MST = 7.36 (± 1.43) days	7	1+2*
	8	3*
	9	1*
	10	1*
Group E		
(Specific F(ab)$_2$-treated)	5	0
N = 9	6	1+1*
MST = 7.22 (0.97)days	7	2+2*
	8	2*
	9	1*

* vascularised grafts

	"t" value	"n"	"p" value
Groups A:B	0.7040	25	> 0.05
Groups A:C	0.7944	24	> 0.05
Groups A:D	3.0102	23	< 0.005
Groups A:E	3.2585	21	< 0.005
Groups D:E	0.2404	18	> 0.05

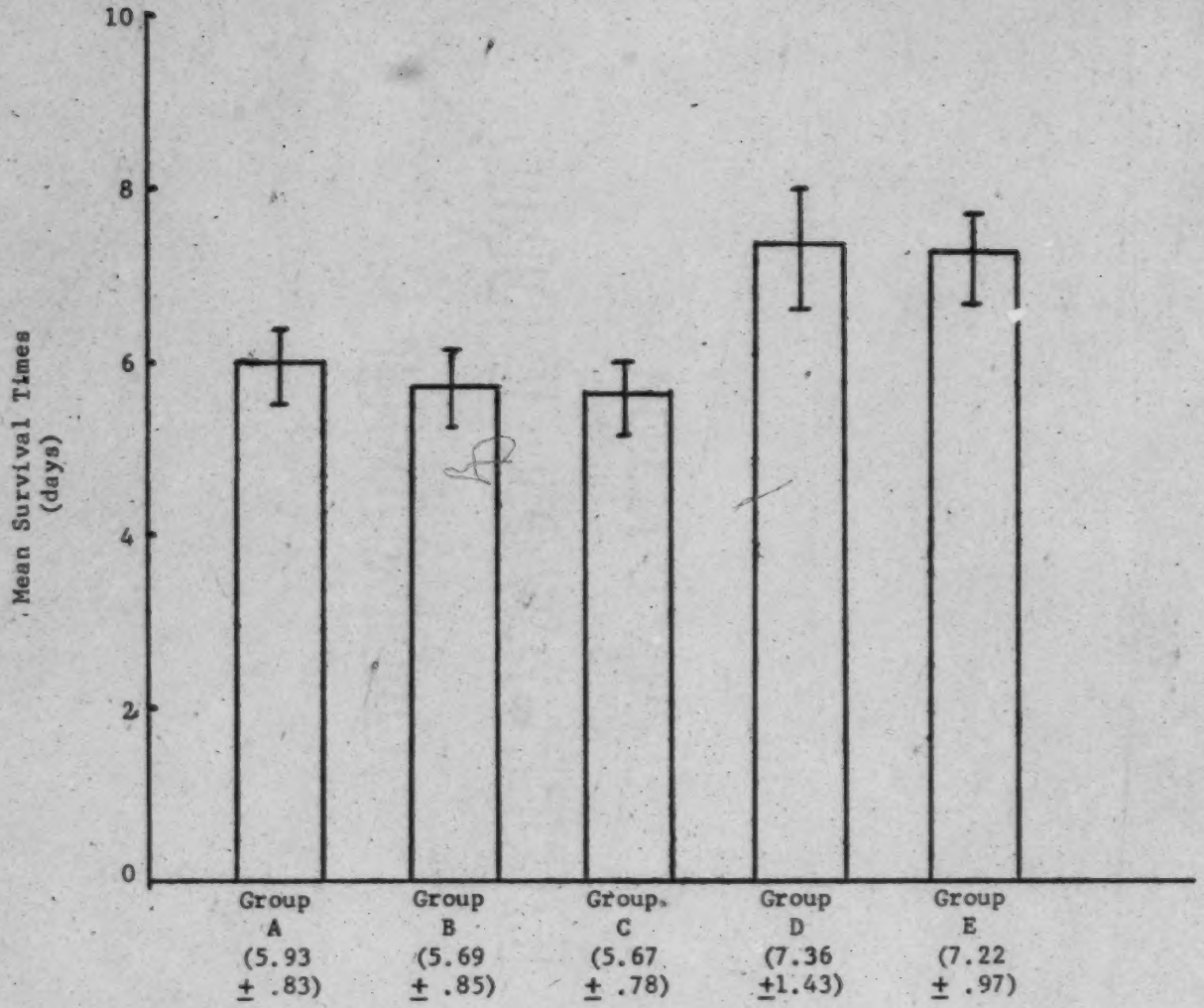


Fig. 8 Rejection times, in days, of skin xenografts after various pretreatment

(Group D) were observed to be vascularised. A similar ratio (6 out of 9) were noted with the specific $F(ab)_2$-treated grafts (Group E). None of the grafts in the control groups became vascularised. If the survival times for the vascularised grafts in Groups D and E were analysed separately from the non-vascularised grafts within the same group, the MST of the vascularised were quite impressive as compared to the control groups (8.5 days compared to 5.9).

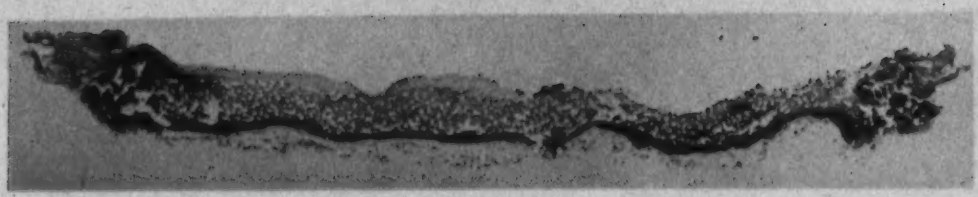
Morphologically, the non-vascularised grafts remained pallid until they turned dark brown and became necrotic. They easily peeled off from the graft beds. In contrast, the vascularised grafts remained pale for about 3 to 4 days, then appeared pink with new blood vessels appearing. Later with the advent of the rejection process, they became edematous, turned purplish and dark blue, rapidly became dry, necrotic and scaly.

Histologically, the non-vascularised grafts showed minimal cellular infiltration - mainly in the subdermal area (that is at the graft-host junction). The epidermis tended to be peeled off and there were generalised necrosis of the epidermal and dermal cells. In contrast, the vascularised grafts were massively and diffusely infiltrated by lymphocytes and mononuclear cells (see Figure 9).

All the rats that were used to raise the antidonor antisera became severely emaciated with the continued immunisation. Six of them died - two from intestinal obstruction secondary to the massive adhesions.

VIII DISCUSSION

Hyperacute rejection is a rare complication in clinical renal transplantation. It is seen mainly in two groups of patients - those receiving kidneys from AB blood group incompatible donors (Starzl



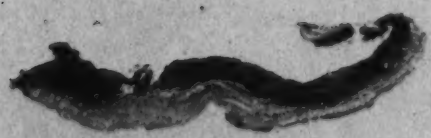


Fig. 9 Histologic features of rejected skin xenograft. Upper, a non-vascularised graft showing minimal cellular infiltration and peeling off of the epidermis. Lower, a vascularised skin xenograft showing the diffuse infiltration and edema.

1964) and in recipients who were presensitised to the donor, either from previous transplants and transfusions or through multiparity (Kissmeyer-Neilsen 1966). Attempts at reproducing experimentally this dramatic form of rejection usually involves the presensitisation of the host to the prospective donor, usually through the repeated exchange of skin grafts, prior to transplanting the kidney. This experimental technique however, takes time, produces inconsistent results and is associated with high morbidity and mortality (Boehmig 1971).

The observation that renal transplants between members of widely disparate species resulted in similar dramatic and vigorous hyperacute rejection (Way 1965, Perper 1966) has led to the suggestion that this may be a convenient and valid model for studying the phenomenon of hyperacute rejection (Kun 1971). The hyperacute rejections of both allografts and xenografts are mediated primarily by preformed antibodies. In both, we have the contributory roles of formed blood elements, changes in coagulation factors and also possibly the release of vasoactive compounds in graft destruction (Boehmig 1971, Rosenberg 1969). Because of the rapidity of the rejection process, going to completion within minutes to hours, the exact role of these subsidiary factors are not known. Interference with any of these factors can, however, lead to graft prolongation. Most attempts at delaying experimental hyperacute rejection are directed at the host with this in mind - to reduce the level of antibody (as with selective plasmapheresis), anticoagulation, diminution of the formed blood elements and giving drugs to counter the action of the vasoactive compounds (see Table I).

An equally fruitful and more convenient approach would be to direct treatment to the grafts with a view of either rendering

them less immunogenic or protecting them from the host's immune effector mechanisms. Ellis (1969) has shown that pretreatment of rabbit kidneys with the telopeptide-poor calf collagen (TPC) resulted in an abrogation of the hyperacute rejection of the kidneys when subsequently transplanted onto a dog. Presumably, the TPC being covalently and specifically bonded onto the endothelial surface of the graft, protects them from the antibody.

The pepsin-digested antibody fragment ($F(ab)_2$) retains its antigen binding capacity but, devoid of the Fc fraction, is unable to bind complement and hence non-cytotoxic (Nisinoff 1971). Antigen-antibody reaction involving the $F(ab)_2$ fragment thus has the effect of covering the antigenic sites without leading to complement fixation and subsequent cytolysis. Such "sterile" combination, however, effectively masks these antigenic sites from subsequent interaction with the intact antibody or immune lymphocytes of the host. Thus Broder (1968) showed that mouse Ehrlich Ascites tumor (EAT) cells incubated with the specific anti-EAT $F(ab)_2$ would grow when subsequently implanted into the Guinea pig. The EAT cells incubated with intact antisera or non-specific $F(ab)_2$ were rejected and failed to grow. Similar pretreatment of monkey kidney allografts with the specific antidonor $F(ab)_2$ effectively delayed their hyperacute rejection when subsequently transplanted into presensitised recipients (Kobayashi 1971). In another model of rejection, dog kidneys perfused with the specific antisera and then re-transplanted into the original host would undergo similar hyperacute rejection (antologous rejections) (Robertshaw 1968). However if they were pre-treated with the $F(ab)_2$ fragment prior to being exposed to the intact antisera, this antologous rejection did not occur (Smith 1971). Our inability to delay the

hyperacute rejection of xenografts with $F(ab)_2$ is disappointing. Four explanations are possible. First, the binding of the $F(ab)_2$ to the antigenic sites was not stable so that with continued xenogeneic perfusion the $F(ab)_2$ fragments were replaced by the intact antibody. An attempt was made at exploring this possibility by tagging the $F(ab)_2$ with fluorescein isothiocyanate and comparing the biopsies taken at various time intervals. However technical difficulties at establishing controls and suitable criteria for comparison made it difficult to interpret. In those experiments where $F(ab)_2$ have been successfully used, only a modest prolongation of survival was obtained and that those kidneys eventually succumb to the full force of hyperacute rejection (Kobayashi 1971).

Second, to be an effective "blocking antibody" the system must contain antibodies for all the antigenic specificities of the graft and in sufficient quantity. There are reasons to suspect our system did not have antibodies for all specificities. It is known that in some species (the dog), the lymphocytes have antigenic specificities which the renal endothelia lack and vice versa (Vetto 1971). Further in rabbits, unlike the mouse and man, the lymphocytes have the lowest concentration of histocompatibility antigens as compared to the liver and kidney (MacDonald 1966). Thus by using lymphocytes to prepare the antisera, we were using a poor and inappropriate source of transplantation antigens. The kidney would have been a better source of antigens in that it will provide more antigen concentration and all specificities. The success obtained with the skin xenograft where the skin was used antigens to raise the antisera underscored this point. A further problem with preparing interspecie

antisera is illustrated by the findings of Sachs (1971). He observed that mice immunised with different strains of rats, and vice versa, will produce antibodies that recognise both specie specificities as well as strain specific histocompatibility antigens. In our experiments, the antidonor antisera were produced by immunising each dog with the lymphocytes of only one rabbit, the prospective donor, thus overcoming the problem of allo- and xeno-specificities of the antisera.

Third possibility is that although there are marked similarities, functionally and morphologically, between the hyperacute rejections of allograft and xenograft, nevertheless there may be fine and quantitative differences such that an observation applicable to one need not be true for the other. This has precedence. Heparin, though effective in delaying the hyperacute rejection of allograft (MacDonald 1970), is completely useless for the xenograft (Hawkins 1971).

The last possibility is that this "peripheral block" is not an important consideration in this transplant system. The observation that, in rabbits, splenectomy done at the time of immunisation completely prevented the development of subsequent hyperacute rejection points to a "central" site of action as being important (Thomas 1972).

The hyperacute rejection of xenografts is a massive and overwhelming process; one that is extremely difficult to overcome. Perhaps the investigation on the role of antibody fragment needs to be evaluated initially on a simpler and easier model, for example a first set renal allograft. Shaipanich (1971) was able to prolong very impressively the survival of first set Fi to ^{P} rat renal allografts by prior treatment with the specific

$F(ab)_2$. Other techniques of producing antidonor antibody, including the possible use of homologous as well as heterologous sources, may be further investigated on this simpler model of rejection.

The results with the skin xenografts were markedly different. The pre-treatment of skin xenografts with the antidonor antisera (ADS) or its $F(ab)_2$ produced not only a quantitative difference in survival rates but also a qualitative difference in their pattern of rejections as judged by the morphologic changes. Untreated grafts were rejected without being revascularised, a pattern similar to the "white graft" reaction of allografts. Grafts treated with ADS or the $F(ab)_2$ were, in most cases, rejected in the conventional pattern - being revascularised first and then rejected. Although the "white graft" reaction is a reflection of heightened sensitivity it is difficult to ascribe a humoral basis for it as the native rat sera lack any antidonor activity as judged by hemagglutinating and lymphocytotoxic titers (Jeekel 1971). This however does not rule out the presence of other destructive antidonor antisera not detected by these two techniques. In contrast, with the hyperacute rejection of kidneys, preformed antidonor antibodies were readily demonstrated (Perper 1966).

The experimental design does not permit one to offer any comments regarding the possible mechanism of action of the specific ADS or its $F(ab)_2$. (See Discussion on Technique). The prolongation of graft survival may be due to a "peripheral" or "central" action of the antibody. If it is assumed to be a peripheral action, then the block must be a transient one as these grafts were eventually rejected. As most of the specific ADS and its $F(ab)_2$-treated grafts were rejected akin to an allograft (that is being first vascularised

and then infiltrated by mononuclear cells) one can postulate that the antibody or $F(ab)_2$ blocks the antigenic sites from the action of the preformed antibodies in the host for a sufficient period to enable one to see the manifestation of cellular immunity. As mentioned in the introductory sections, most of the cross-specie rejections are humoral-mediated and occur very rapidly so that the slower CMI reaction does not have time to become manifested. Thus by blocking the action of the humoral antibody and allowing the grafts to survive longer, the action of cellular immunity becomes apparent. Alternatively, it may be possible that incubation with the ADS or $F(ab)_2$ resulted in antigenic alterations in the grafts such that a new immune response will have to be initiated. This concept of antigenic alteration is convincingly illustrated by experiments on autologous skin rejections. Cohen (1969) and Brautbaur (1969) showed that the incubation of skin grafts with immuned xenogeneic sera or their temporary transplantation onto a xenogeneic host will result in the rejection of these grafts when subsequently retransplanted onto the original hosts. The humoral and/or cellular factors must have brought about sufficient antigenic alterations on the grafts to be recognised as "foreign" by their original hosts.

A central mechanism cannot be ruled out. It may be argued that some of the ADS or $F(ab)_2$ were physically adherent to the grafts and subsequently absorbed systemically by the host. However, the amount of antisera absorbed in this way must be minimal and inconsequential. Jeekel (1971), also working with mouse-to-rat skin grafts, showed that relatively large doses of ADS need to be given systemically to effect any prolongation of graft survivals. ADS given in range of

0.3 to 0.5 ml for three days only were ineffective. However this "central"effect could have been easily ruled out by a simple modification of the experimental design. Thus two grafts, one treated, the other a control, could have been placed on the same recipient - a "central" effect would influence the survival of both grafts; a "peripheral" one would influence only the treated graft.

Referring back to Jeekel's work, it is remarkable that he was able to prolong very impressively (up to 38 days) the survival of skin xenografts by the prolonged systemic administration of ADS. Although he did not comment on the morphologic changes of these grafts it is unlikely that they could have survived that long without neovascular support. Also an interesting observation was that minor differences in the technique of producing the antisera resulted in marked variable efficiency. Thus sera obtained less than two weeks after the last immunisation and those immunised without the use of Freund's adjuvants were ineffective. The technique used in the present study differs from Jeekel's in three ways. One, the ADS was homologous (actually autologous, as it was raised in the same syngeneic strain of rats as the recipients). Two, spleen cells plus skin were used as antigens. The spleen cells were used as in the mouse they provide a good source of transplantation antigens (Kahan 1969); the skin, to provide any additional antigenic specificities not found in the lymphocytes. Three, the sera were harvested one week after the last immunisation. Very high (1:1024) ADS titers was obtained with this technique.

The efficiency of both the ADS and $F(ab)_2$ is interesting and cannot be readily explained. The non-cytotoxicity of the intact

antibody cannot be explained in terms of complement deficiency. While it is true that certain strains of mice and rats may lack certain components of the complement system (Snell 1966), this particular strain of rat (Lewis) does not (Chavez-Peon 1971). Whether or not destructive effect follows an antibody-antigen reaction depends on a number of factors - the type of target cells (solid or ascitic), and the ability of the Ab-Ag complex to trigger the complement system. The sequence of complement reaction chain is very complex and it is to be expected that there may be specie-differences in the concentration of the various components and the ease with which they can be triggered. It is likely that this technique of raising ADS truly produces an enhancing antisera; pepsin-digestion then is not expected to alter this property.

In the intact animal, the rejection of skin xenograft is a function of both humoral and cellular immunity and that depending on the circumstances one predominates over the other. Lance's work on the induction of tolerance in mice to rat cells, showed that while in non-tolerant (that is non-thymectomised) mice the rejection of rat skin xenografts is accompanied that by high titers of antirat antibodies, in the tolerant, thymectomised animals the rat skin survives despite the presence of such antibodies (Lance 1969). One is reminded of the earlier work by Good, who demonstrated the delayed rejections of skin grafts in agammaglobulinemic patients despite the fact that these patients have intact cellular immunity responses (Good 1957). There is thus a dependency on each other and synergism between the cellular and humoral responses.

Discussion on Technique

Renal Xenografts. I had used the technique of *in vivo* xenogeneic perfusion rather than actually transplanting the kidneys for two reasons. First by merely cannulating rather than anastomosing the vessels, the operative, and hence anoxia, time was markedly shortened. Second, by not taking the kidneys out I had purposely avoided manipulating them. It is known that undue or excessive manipulation of the kidney can lead to reflex arteriolar spasm which may significantly decrease the perfusion of the organ. Since the criterion for rejection was dependent on the blood flow through the graft, any extraneous factors that can influence blood flow must be minimised.

A major criticism of the experimental technique is the fact that I did not show that the antidonor $F(ab)_2$ was actually bound to the antigenic sites on the grafts. It was merely assumed that half an hour of perfusion time was adequate for the antigen-antibody reaction to take place. Smith's work (1970) with the hyperacute rejection in dogs and Kobayaski's (1971) in monkeys indicated that this was sufficient time. I attempted to test this out by labeling the $F(ab)_2$ with the fluorescein isothiocyanate. In two out of four perfusions in Group E in which this was done, fluorescein was indeed detected diffusely in the glomeruli and small blood arterioles. However the interpretation was clouded by the fact that I could not establish adequate control studies and difficulty in differentiating between background and non-background staining.

A further criticism may be directed at the technique of raising the antidonor antisera. As indicated earlier, in rabbits

the lymphocytes are a poor source of transplantation antigens. Perhaps the antisera could have been better prepared by sensitising the dog through repeated skin grafts as with the experiments on hyperacute rejection of allografts.

Skin Xenografts. Unlike renal xenografts where, by using an objective criterion as the graft's blood flow, the end-point of rejection could be easily defined to the minute, the end-point for skin xenograft rejection is less easily determined. The criteria for rejections are subjective, dependent upon the slow gross morphologic changes. Hence variations in interpretations are to be expected with different observers and with the same observer on different occasions. This problem of interpretation is further compounded by the fact that differences in survival of a few days can be very sfgnificant biologically, apart from the statistical significance. As an example, the prolongation of skin allografts in recipients treated with antilymphocytic globulin is only a matter of days, at most a week; but the same treatment with renal allografts resulted in prolongation in terms of months (Russel 1970). It is just that the skin graft is a sensitive assay in transplantation system.

I had attempted to overcome the problem of interpretation in three ways. First, a control autograft was placed on the opposite side of the animal for direct comparison. Second, an independent observer, another research fellow not involved in the project, was asked to interpret the grafts in a blind fashion. Third, the skin grafts in the various groups were done in two batches - half of each group was done at about the same time in few successive days.

Two further refinements in techniques may be suggested. One, a much bigger number of animals in each group may be used so that small differences in survival rates may be detectable. Initially it was aimed to have about 15-20 animals to each group, but difficulties in preparing adequate quantity of the sera and few technical failures in grafting reduced the number to about a dozen. Two, a more objective criterion for rejection could be used. Steinmuller (1962) has suggested that the diagnosis of rejection be made on histologic findings rather than gross features. His technique involves the daily biopsying of sample grafts and counting the percentage of viable dermal cells - end-point being when 80% of the cells were dead. I failed, however, to see how this elaborate technique being an improvement. To interpret viable cells from dead ones from conventionally-stained slides can be quite difficult and subjective. Besides, the frequent biopsying on the graft could be a factor in graft failure.

Lastly, as has been discussed earlier, the experimental design merely provides an interesting observation and maybe suggest, but not establish, a mechanism to explain the phenomenon. Two simple modifications in the protocol may help to elucidate the mechanism of action - in particular a 'peripheral' versus a 'central' effect. Thus two grafts, one pretreated and the other non-treated and serving as a control, may be placed on the same recipient. A central effect would result in a prolongation of both grafts; a peripheral one would influence only the pretreated graft. Additionally the effect of systemic administration of the ADS and $F(ab)_2$ may be compared with those receiving local treatment.

D. Conclusion and Summary

1. Pretreatment of xenografts with antidonor antisera (ADS) or $F(ab)_2$ prolonged the survival of mouse-to-rat skin xenografts but not that of rabbit-to-dog renal xenografts.

2. Skin xenografts that were pretreated with ADS or $F(ab)_2$ not only survived longer but also became vascularised prior to being rejected; in contrast, control grafts were rejected in a 'white graft' fashion without being vascularised.

3. The failure to prolong the renal xenografts by prior treatment with ADS or $F(ab)_2$ may be due to the inappropriate technique of preparing the ADS and the overwhelming nature of the hyperacute rejection.

4. Two major limitations and criticisms of the experiments are: one, the ADS or $F(ab)_2$ had not been shown to be attached to the grafts' antigenic sites during the preincubation period and two, the blocking activity of the antidonor $F(ab)_2$ had not been assayed *in vitro* using target donor lymphocytes except in the two dogs in the renal xenograft experiment.

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X APPENDIX

Table V

RENAL BLOOD FLOW: GROUP A (SALINE-TREATED)

Time (Mins)	Kidney #1 ml/min	Kidney #1 %*	Kidney #2 ml/min	Kidney #2 %*	Kidney #3 ml/min	Kidney #3 %*	Kidney #4 ml/min	Kidney #4 %*	Kidney #5 ml/min	Kidney #5 %*	Kidney #6 ml/min	Kidney #6 %*	Mean % Flow
1	3.8	100	3.8	100	3.0	100	9.8	100	3.2	100	5.0	100	100
2	4.8	126	3.8	100	3.0	100	10.4	106	1.8	56	4.8	96	97 (23)
3	5.0	132	3.6	95	3.6	120	9.8	100	1.2	36	4.8	96	97 (32)
4	5.4	142	4.2	111	3.8	127	8.4	86	1.2	38	4.5	90	99 (37)
5	4.6	121	4.8	126	3.8	127	6.7	68	1.1	34	4.5	90	94 (38)
6	3.6	95	5.2	147	3.4	113	5.4	55	0.8	25	1.1	44	80 (46)
7	3.4	89	2.4	63	2.2	73	4.4	45	0.6	19	1.1	44	55 (25)
8	2.1	55	2.0	53	2.5	83	3.6	37	0.5	16	0.9	18	44 (25)
9	1.5	39	2.0	53	1.0	33	3.0	31	0.2	6	0.5	10	27 (18)
10	0.6	16	1.3	34	0.8	27	1.5	15	0.2	6	0.3	6	17 (11)
11	-		0.5	9	0.9	27	-		-		-		
Rejection Time (mins)	10		11		11		10		7		8		9.5(1.64)
Comments	-		Urine Flow		-		-		-		Urine Flow		

%* - Per cent of initial blood flow

Table VI

RENAL BLOOD FLOW: GROUP B (NON-SPECIFIC SERA-TREATED)

Time (Mins)	Kidney #1 ml/min	Kidney #1 %*	Kidney #2 ml/min	Kidney #2 %*	Kidney #3 ml/min	Kidney #3 %*	Kidney #4 ml/min	Kidney #4 %*	Kidney #5 ml/min	Kidney #5 %*	Kidney #6 ml/min	Kidney #6 %*	Mean % Flow
1	4.2	100	2.0	100	7.4	100	5.6	100	5.4	100	7.2	100	100
2	4.0	95	3.2	160	8.2	111	4.2	75	5.2	96	6.4	89	104 (30)
3	3.6	86	3.5	180	6.5	88	3.0	54	3.4	63	6.5	90	94 (45)
4	3.8	90	3.2	160	4.9	66	2.5	45	3.0	56	5.6	78	83 (41)
5	3.0	71	3.2	160	2.0	27	1.8	32	2.9	58	4.2	58	68 (48)
6	2.2	52	3.8	190	1.0	14	1.6	29	2.6	48	3.0	42	62 (62)
7	2.6	62	1.8	90	0.6	8	1.2	21	2.0	37	2.5	35	42 (30)
8	2.6	62	1.4	70	0.6	8	0.7	13	2.4	44	1.7	24	37 (26)
9	2.8	67	1.3	63	0.6	8	0.6	11	1.4	26	1.3	18	32 (26)
10	2.5	60	0.9	45	0.7	9	-		0.6	11	0.8	11	-
11	0.7	16	0.6	30	-				-		0.2	3	
Rejection Time (Mins)	11		12		6		7		10		9		9.2 (2.32)
Comments					Urine Flow		Urine Flow				Urine Flow		

%* - Per cent of initial blood flow

Table VII

RENAL BLOOD FLOW: GROUP C (NON-IMMUNE F(ab)$_2$-TREATED KIDNEYS)

Time (Mins)	Kidney #1 ml/min	Kidney #1 %*	Kidney #2 ml/min	Kidney #2 %*	Kidney #3 ml/min	Kidney #3 %*	Kidney #4 ml/min	Kidney #4 %*	Kidney #5 ml/min	Kidney #5 %*	Mean % Flow
1	20.0	100	4.6	100	2.6	100	6.5	100	3.2	100	100
2	21.0	105	6.8	148	1.7	65	5.6	86	3.8	119	104 (32)
3	13.2	66	8.6	187	1.7	65	4.2	65	3.6	113	99 (53)
4	9.0	45	8.6	187	1.0	38	3.0	46	3.2	100	83 (63)
5	5.0	25	8.0	174	1.6	30	2.5	38	3.0	94	72 (63)
6	2.0	10	7.0	152	0.8	30	1.8	28	3.0	94	63 (59)
7	1.0	5	6.4	139	0.9	37	1.6	25	2.7	84	58(50)
8	-	-	4.4	96	0.9	37	1.2	18	1.5	47	- (-)
9			4.0	87	0.6	23	1.0	15	0.8	25	- -
10			2.4	52	0.5	19	0.6	9	0.6	19	- -
			1.4	30	-	-	-	-	-	-	- -
			1.0	22							
			-	-							
Rejection Time (Mins)	6		12		9		8		10		9.00(2.23)
Comments	-		-		Urine Flow		-		-		

%* - Per cent of initial blood flow

Table VIII

RENAL BLOOD FLOW: GROUP D (IMMUNE SERA-TREATED KIDNEYS)

Time (Mins)	Kidney #1 ml/min	Kidney #1 %*	Kidney #2 ml/min	Kidney #2 %*	Kidney #3 ml/min	Kidney #3 %*	Kidney #4 ml/min	Kidney #4 %*	Mean % Flow
1	9.4	100	2.2	100	2.5	100	7.2	100	100
2	9.6	102	2.1	95	2.6	104	7.8	108	102 (5)
3	8.0	85	2.0	90	2.3	92	6.5	90	89 (3)
4	7.0	74	1.5	68	2.0	80	5.7	79	75 (5)
5	3.3	35	0.5	23	1.8	72	3.2	44	43 (2)
6	2.0	21	0.5	23	2.1	84	2.5	35	41 (29)
7	0.6	6.4	-	-	1.5	60	2.8	39	26 (28)
8	0.2	2			1.3	52	0.8	11	16 (24)
9	-	-			1.0	40	0.5	7	
10					0.6	24	-	-	
Rejection Time (Mins)	6		5		9		7		6.75(1.7)
Comments	Hematuria		-		-		-		

%* - Per cent of initial blood flow

Table IX

RENAL BLOOD FLOW: GROUP E (IMMUNE F(ab)$_2$-TREATED KIDNEYS)

Time (Mins)	Kidney #1 ml/min	Kidney #1 %*	Kidney #2 ml/min	Kidney #2 %*	Kidney #3 ml/min	Kidney #3 %*	Kidney #4 ml/min	Kidney #4 %*	Mean % Flow
1	7.4	100	10.4	100	6.0	100	3.4	100	100
2	5.0	68	8.8	85	7.0	117	3.7	109	95(22)
3	2.4	32	5.2	50	6.4	106	3.0	88	69(34)
4	2.0	27	2.2	21	6.0	100	2.9	85	58(40)
5	1.0	14	2.0	19	5.6	94	2.5	74	50(40)
6	1.0	14	0.8	8	2.2	37	1.8	53	28(21)
7	0.8	11	0.8	8	0.8	13	1.7	50	21(20)
8	0.6	8	0.9	10	0.2	3	0.9	26	12(10)
9	-	-	0.6	6	-	-	0.7	21	
10			-	-			0.4	12	
							0.2	6	
Rejection Time (Mins)	5		4		7		9		6.25(2.2)
Comments	Hematuria								

%* - Per cent of initial blood flow

Table X

ARTERIOVENOUS GRADIENT OF FORMED BLOOD ELEMENTS AT 5 MINS.

Experimental Group	Platelet ($x10^3/min^3$)			WCB ($x10^3/min^3$)			RBC ($x10^6/min^3$)		
	A	V	$(\frac{A-V}{A})\%$	A	V	$(\frac{A-V}{V})\%$	A	V	$(\frac{A-V}{A})\%$
Group A	314	20	94	7.3	5.4	26	3.27	4.14	- 4
(saline treated)	320	14	96	8.8	6.6	25	3.07	4.14	-35
	346	162	53	10.8	8.6	21	7.14	6.65	7
	274	30	89	18.7	7.9	58	-	-	-
	142	100	30	13.1	1.9	85	4.71	3.65	23
	202	35	83	6.0	3.6	40	4.00	4.16	- 4
			74 (27)			43 (25)			
Group B	200	78	61	15.4	11.1	28	5.65	5.60	9
(non-specific sera)	396	38	90	6.5	4.4	32	3.96	3.46	13
	274	30	88	18.7	7.9	58	-	-	
	244	10	96	5.3	4.4	18	3.74	3.35	10
	142	120	21	13.1	2.0	85	4.71	3.63	23
	372	22	94	7.8	3.1	60	4.82	4.36	10
			75 (29)			47 (25)			13 (6)
Group C	280	76	73	7.2	7.0	28	3.88	3.60	7
(non-specific $F(ab)_2$)	248	10	96	9.3	6.3	32	4.07	3.20	21
	216	74	66	11.7	7.7	34	3.23	3.58	-11
	230	28	88	14.2	2.4	83	4.75	3.68	23
	312	26		8.6	2.5		-	-	-
			83 (13)			50 (25)			
Group D	294	42	86	9.8	6.5	34	4.78	4.74	0.5
(Immune-sera treated)	438	60	86	8.3	4.7	43	5.47	4.18	24
	244	10	96	5.3	3.5	35	3.86	3.74	3
	236	36	85	4.0	3.1	23	3.74	3.24	13
			88 (5)			34 (8)			10 (11)
Group E	432	90	79	8.3	6.5	22	4.44	4.54	- 3
(Immune $F(ab)_2$-treated)	108	40	63	9.8	2.7	72	4.55	3.23	29
	384	76	80	11.3	4.9	57	5.07	4.12	19
	384	100	74	11.3	6.4	43	5.07	5.30	- 5
			74 (8)			49 (21)			

Microlymphocytotoxicity Assay Technique

The lymphocytotoxicity titers of the dog sera were assayed by Dr. P. McConnachie using the Terasaki's microdroplet technique and Guinea pig sera as source of complement (Mittal et al: Transplantation 6:913, 1968).

The native dog serum has antirabbit lymphocytotoxic titer of 1/8; the pepsin-digested sera showed strongly positive (over 80% cell death) reaction at single and double strengths. Interestingly, incubation of rabbit lymphocytes with autologous rabbit sera and dog sera resulted in reduction of the dog's cytotoxic titer.

The lymphocytotoxic titers of the immune sera were determined in two dogs - they were $1:10^2$ and $1:10^4$; their titers for the corresponding $F(ab)_2$ (adjusted to protein concentration of 2-3 G/100 ml) were strongly positive at single, double and triple strengths. The blocking activity of the specific $F(ab)_2$ was tested in vitro using non-donor rabbit lymphocytes by incubating, without complement, with the $F(ab)_2$ followed by the ADS with complement. There was a reduction in titers from $1:10^2$ to 1:2 and $1:10^4$ to 1:8. Thus blocking activity was demonstrated in vitro despite using non-donor rabbit lymphocytes.

The lymphocytotoxicity titer of the rat sera were not assayed.